Mannose 6-Phosphate Receptor Expression in Inflammation

by

Elizabeth J. Hindmarsh, B.Sc. (Hons)

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Division of Cell Biology
John Curtin School of Medical Research
Australian National University
Canberra, Australia

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STATEMENT

I certify that, except for the preparation of ascites fluid (Section 3.2.6) and treatment of rats with mAb 1G7/9H4 (section 3.3.5) which were done by Dr D. Willenborg, the purification of mAb 1G7/9H4 (Section 3.2.7) which was done by Jenny Swan, and one rat T cell adhesion assay (Section 5.3.3) and the treatment of CD3-stimulated T cells with chemokines (Section 5.3.4) which were done by Karen Jakobsen, all experiments described in this thesis represent my own work, were done by me, and have not been previously submitted for a degree at this or any other university.

Elizabeth Hindmarsh

Elizabeth J. Hindmarsh

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ABBREVIATIONS

BP	basic protein
cAMP	cyclic AMP
CD	cluster designation
CLA	cutaneous lymphocyte-associated
CNS	central nervous system
Con A	conconavalin A
CS	castanospermine
DELFIA	dissociation-enhanced lanthanide fluoroimmunoassay
DMEM	Dulbecco's Modified Eagle Medium
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FIU	fluorescence intensity units
fl-PPME	fluorescein-PPME conjugate
F1P	fructose 1-phosphate
GAG	glycosaminoglycan
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor
GNAc	N-acetylglucosamine
HAT	hypoxanthine-aminopterin-thymidine
HBSS	Hank's balanced salt solution
HEV	high endothelial venule
HSPG	heparan sulfate proteoglycan
HT	hypoxanthine-thymidine
HuTSP-1	human T cell-specific serine proteinase
HUVE cell	human umbilical vein endothelial cell
Ig	immunoglobulin
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
IL-1	interleukin 1
IL-8	interleukin 8
IFN-γ	interferon γ
i.p.	intraperitoneal
i.v.	intravenous

LDL	low density lipoprotein
LPS	lipopolysaccharide
mAb	monoclonal antibody
MALT	mucosa-associated lymphoid tissue
Man	mannose
MBP	myelin basic protein
MCAF	monocyte chemotactic and activating factor
MCP-1	monocyte chemotactic protein 1
MEI	MPR-300 expression index
MIP-1α	macrophage inflammatory protein-1α
MIP-1β	macrophage inflammatory protein-1β
MLN	mesenteric lymph node
MMP	matrix metalloproteinase
M6P	mannose 6-phosphate
MPR	mannose 6-phosphate receptor
MPR-46	46 kDa mannose 6-phosphate receptor
MPR-300	300 kDa mannose 6-phosphate receptor
M_r	relative molecular weight
MSTP-1	murine T cell-specific serine proteinase
PA	plasminogen activator
PAF	platelet activating factor
PAGE	polyacrylamide gel elctrophoresis
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PBST	0.05% Tween 20/PBS
PE	phycoerythrin
PECAM-1	platelet/endothelial cell adhesion molecule
PEG	polyethylene glycol
PHA	phytohaemagglutinin
PLN	peripheral lymph node
PMA	phorbol myristate acetate
PMP	pentamannose phosphate
PMSF	phenylmethylsulfonylfluoride
PN-1	protease nexin-1
PPME	phosphomannan monoester core
RANTES	*r*egulated upon *a*ctivation, *n*ormal *T e*xpressed, and presumably *s*ecreted
RBC	red blood cell
RER	rough endoplasmic reticulum
RT	room temperature

SDS	sodium dodecyl sulfate
TGN	*trans*-Golgi network
TIMP	tissue inhibitor of matrix metalloproteinases
TNF	tumour necrosis factor
TPBS	0.5% Triton X-100 in PBS
uPA	urokinase-type plasminogen activator
uPA-R	urokinase-type plasminogen activator receptor
VCAM-1	vascular cell adhesion molecule-1

ABSTRACT

Migration of circulating leukocytes into extravascular tissues is required for the effective functioning of the immune system, being essential to such processes as inflammation, and lymphocyte recirculation between the blood, secondary lymphoid tissues and non-lymphoid tissues. Extravasating leukocytes first adhere to the lumenal surface of the vascular endothelium, migrate between (or through) the endothelial cells, and penetrate the subendothelial basement membrane.

Leukocyte-endothelial adhesive interactions have been well characterised at the molecular level, however the mechanisms by which leukocytes penetrate the subendothelial basement membrane are less clear. Several studies suggest that activated leukocytes secrete hydrolytic enzymes which degrade the basement membrane. Following evidence that cell surface localization of degradative enzymes may be an important means of regulating ECM degradation by metastatic tumour cells, and that lysosomal enzymes can be expressed on the cell surface via mannose 6-phosphate receptors (MPRs), Parish and coworkers proposed that leukocyte extravasation may be dependent on the cell surface expression of degradative lysosomal enzymes. They subsequently showed that mannose 6-phosphate (M6P) and castanospermine, both inhibitors of the lysosomal enzyme-MPR interaction, are potent inhibitors of passively-induced experimental autoimmune encephalomyelitis (EAE) and adjuvant arthritis in rats. As development of disease in these animal models is dependent on the passage of transferred T cells from the circulation into the appropriate tissue, the anti-inflammatory effects of these compounds were proposed to result from their ability to prevent the binding of lysosomal enzymes to cell surface MPRs on extravasating T cells. The aim of this thesis was to test the hypothesis, proposed by Parish *et al.* (1990), that cell surface expression of lysosomal enzymes via MPRs contributes to degradation of the subendothelial basement membrane by extravasating leukocytes.

The MPRs include two receptors, designated MPR-300 and MPR-46 on the basis of M_r, which are expressed predominantly on intracellular membranes, with approximately 10% at the plasma membrane. They recognise a M6P marker common to all lysosomal enzymes, and mediate their transport to lysosomes by both an intracellular and endocytic pathway. Newly synthesized lysosomal enzymes are bound by MPR-300 and MPR-46 in the Golgi apparatus, and transported to a prelysosomal compartment, from which they are packaged into lysosomes. Enzymes may also be secreted, either through failure to bind MPRs in the Golgi, or directly by means of MPR-46. Extracellular lysosomal enzymes can be recaptured and endocytosed by MPR-300 expressed at the cell

surface. Secretion of lysosomal enzymes from the cell, and subsequent binding to cell surface MPR-300, are thus central to our hypothesis.

If MPR-300 is involved in the cell surface expression of degradative enzymes by extravasating leukocytes, then disruption of ligand binding to this receptor should reduce the ability of these cells to degrade the subendothelial basement membrane, by analogy with the proposed action of M6P in EAE and adjuvant arthritis. The first part of this study aimed to specifically prevent lysosomal enzyme binding to cell surface MPR-300, using monoclonal antibodies (mAbs) specific for this receptor. Chapter 2 describes an assay for detecting cell surface MPR-300, based on the binding and endocytosis of a fluorecein-labelled, M6P-rich polysaccharide (PPME). This assay was used to identify a rat cell line expressing MPR-300 on the plasma membrane, for use in screening hybridoma supernatants for antibodies specific for the extracellular portion of MPR-300. As this assay was dependent on binding of a M6P-bearing ligand, it also established a method for screening antibodies for their ability to inhibit ligand binding to MPR-300. In Chapter 3, a panel of 12 monoclonal antibodies specific for the extracellular portion of rat MPR-300 were produced. Using the PPME binding and uptake assay, their ability to disrupt ligand binding to cell surface MPR-300 was assessed. None interfered with ligand binding to a significant degree, precluding their use in examining the consequences of inhibiting cell surface binding of lysosomal enzymes on the ability of T cells to induce EAE *in vivo*, or to degrade ECM components *in vitro*.

The second part of this study involved a comparison of the cell surface expression of MPR-300 in resting and activated T cells. As activated, but not resting, T cells enter the central nervous system (CNS) and induce EAE, Parish and coworkers proposed that the cell surface expression of lysosomal enzymes may be increased by activation, thus contributing to the enhanced invasive capacity of these cells. Lysosomal enzyme expression at the cell surface could be increased through an activation-induced upregulation of MPR-300 on the plasma membrane, thus providing additional binding sites for extracellular enzymes. In addition, the extracellular pool of lysosomal enzymes available for binding could be raised through an increase in the expression of MPR-46 in the Golgi, as this receptor participates in the secretion of newly synthesized lysosomal enzymes.

The study described in Chapter 4 compared the cell surface expression of MPR-300 on resting and activated T lymphocytes from a variety of sources, using one of the mAbs specific for rat MPR-300 prepared in Chapter 3. No MPR-300 was detectable on the cell surface of rat lymphocytes from secondary lymphoid

tissues or the peritoneal cavity, or on rat and human peripheral blood leukocytes, with the exception of human neutrophils. Expression of MPR-300 was induced on the plasma membrane of rat T cells by several activating stimuli. MPR-300 was expressed on the surface of splenic T cells after 3-4 days stimulation with Con A, although similar treatment did not affect human peripheral blood T cells. MPR-300 was also detected on the plasma membrane of activated T cells present in a thioglycollate-induced inflammatory peritoneal exudate. These observations demonstrate that unstimulated T cells do not express MPR-300 at the cell surface, and support the proposal that it's expression can be induced by activating stimuli, consistent with the hypothesis that expression of lysosomal enzymes on the plasma membrane of activated T cells facilitates their degradation of the subendothelial basement membrane and entry into extravascular tissues.

Endothelial cells are known to provide activating signals to adherent leukocytes, producing such effects as integrin-mediated tight binding, which enhance their capacity to extravasate and enter the extravascular tissue. In Chapter 5, endothelial cells were examined for their ability to induce MPR-300 expression on the plasma membrane of adherent T cells. Expression of MPR-300 at the surface of T lymphocytes, both resting and Con A-stimulated, was assessed after adhesion to endothelial cell monolayers. Neither resting or stimulated human peripheral blood T cells expressed MPR-300 initially, and adhesion to human umbilical vein endothelial (HUVE) cells had no affect on the resting T cells. Con A-stimulated cells, however, showed a marked, transient increase in MPR-300 expression after adhesion, demonstrating that endothelial cells can induce the upregulation of MPR-300 on T cells. The inability of endothelial cells to induce MPR-300 expression on resting cells suggested that two signals are required, such that interaction with endothelium provides a second signal to cells which have been pre-activated.

This suggested a re-evaluation of the conclusion drawn in Chapter 4, that activating stimuli directly induce expression of MPR-300 on the surface of T lymphocytes. A requirement for both a pre-activating and an endothelial-derived signal is compatible with MPR-300 expression by activated peritoneal exudate lymphocytes, as these cells entered the peritoneal cavity from the circulation and so have had the opportunity to interact with the vascular endothelium. It can also be reconciled with the expression of MPR-300 on Con A-stimulated rat T cells. Interaction with rat brain microvascular endothelium did not induce further MPR-300 expression on these cells, suggesting that human and rat T cells may interact differently with Con A, such that Con A

supplied the rat T cells with both the activating and otherwise endothelial-derived signals.

The demonstration that endothelial cells can induce the upregulation of MPR-300 on pre-activated T cells suggests a modification to the hypothesis proposed by Parish *et al.* (1990). Expression of MPR-300 does appear to be restricted to recently activated T cells, consistent with the proposal that the activated, more invasive phenotype corresponds with the ability to express lysosomal enzymes at the cell surface, however activation alone may be insufficient to induce this change. Activation may prime circulating T cells, enabling them to later upregulate MPR-300 expression in response to an endothelial-derived signal. This is an attractive possibility as it correlates with the increased invasive potential of activated T cells, but restricts cell surface expression of MPR-300, and potentially lysosomal enzymes, to T cells in contact with the vascular endothelium. This limits the ability to degrade the subendothelial basement membrane by a lysosomal enzyme-dependent mechanism to T cells that have commenced the process of extravasation.

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Migration of leukocytes from the bloodstream into extravascular tissues is essential for the effective functioning of the immune system. A principal function of the immune system is to protect the host against microbial invasion, and its defensive role involves continuous surveillance of lymphoid and non-lymphoid tissues for the presence of foreign antigen, followed by proliferation and differentiation of antigen-specific cells, and dissemination of effector cells to sites of antigen exposure. Lymphocytes are the major participants in this process, as they determine the specificity of the immune response, and their response to antigen orchestrates its effector limb. Naive lymphocytes constantly recirculate between the blood and the secondary lymphoid tissues, an important means of exposing potentially reactive lymphocytes to the full spectrum of antigen entering the body. Once activated, effector or memory lymphocytes also enter peripheral tissues in small numbers, presumably for the purpose of immune surveillance, as well as in response to low level antigenic challenges. The most spectacular induction of leukocyte migration into tissues, however, occurs during an inflammatory response, in which neutrophils, monocytes and lymphocytes migrate into injured or infected tissues on a large scale. Leukocyte migration also contributes to some less desirable effects of the immune response, such as chronic inflammation, cell-mediated autoimmune disease and graft rejection (Vachula and Epps, 1992).

Leukocyte migration into extravascular tissues has been studied largely in the context of lymphocyte recirculation, and inflammation, the major features of which are outlined below.

1.2 Leukocyte migration into extravascular tissues

1.2.1 Lymphocyte recirculation

Lymphocytes recirculate continuously through the various tissues of the body, entering from the blood and leaving via the lymph. Since very few lymphocytes recognise any specific antigen, this constant movement is necessary to give the entire range of receptor specificities the opportunity to contact antigen. Lymphocyte migration also disseminates the effector and memory cells produced in a local immune response to other regions of the body, ensuring systemic immunity. The recirculation pathway is dependent on the cell type and its differentiation state. B cells, for example, migrate preferentially to mucosa-associated lymphoid tissues, while T cells favour

peripheral lymph nodes. Naive cells recirculate between all secondary lymphoid tissues, but once activated, effector and memory cells also migrate to non-lymphoid sites in a highly tissue-specific manner. This field has been reviewed recently by Butcher (1986), Duijvestijn and Hamann (1989), Mackay (1991), Mackay (1992), (Mackay (1993a), Picker and Butcher (1992), and Yednock and Rosen (1989).

Naive or virgin lymphocytes preferentially migrate to secondary lymphoid tissues (Mackay, 1991), such as the spleen, peripheral lymph nodes (PLNs) and non-encapsulated mucosa-associated lymphoid tissues (MALT; including the Peyer's patches in the lamina propria of the small intestine, tonsils in the pharynx, and lymphoid follicles in the appendix and upper airways) (Abbas *et al.*, 1991). The PLNs and MALT are the termination point of the afferent lymphatics which drain lymph from most tissues, carrying with it antigens that have entered the body through skin or mucosal surfaces, while the spleen filters the blood, accumulating antigens that have entered the vascular system (Picker and Seigelman, 1993). These lymphoid tissues thus act as a collection point for both lymphocytes and antigens, bringing them together in the presence of specialized antigen presenting cells in an environment suited to the activation of naive lymphocytes (Mackay, 1993a). Lymphocytes enter lymphoid tissues across high endothelial venules (HEV), specialized regions of the vasculature which selectively mediate high levels of extravasation . After about 18-20 h in the lymph node, lymphocytes that fail to contact antigen leave with the efferent lymph and return to the bloodstream via the thoracic duct (Mackay, 1991). Naive cells are thought to recirculate between the various secondary lymphoid tissues until they either respond to their specific antigen or die.

Lymphocytes that encounter specific antigen within the lymphoid tissues differentiate into effector or memory cells, and return to the circulation with markedly different recirculation properties. Firstly, they migrate to non-lymphoid tissues, both constitutively at very low levels (Janossy *et al.*, 1989; Mackay *et al.*, 1990; Rannie and Donald, 1977), and at much higher levels during inflammation (Pitzalis *et al.*, 1988; Pitzalis *et al.*, 1991; Saltini *et al.*, 1990). Secondly, homing is tissue-specific, with distinct subsets of cells recirculating through particular organs or tissues; this preference may be for the tissue/lymphatic bed in which they were first activated (Picker and Butcher, 1992). Many investigators have shown two distinct pools of recirculating lymphocytes, one associated with peripheral lymph nodes,and the other with

gut-associated lymphoid tissues (Peyers patches and mesenteric lymph nodes). Lymphoblasts obtained from PLNs and injected intravenously into recipient animals return preferentially to the PLNs (Griscelli *et al.*, 1969; McDermott and Bienenstock, 1979), whereas lymphoblasts from the thoracic duct, mesenteric lymph nodes or intestinal lymph migrate to gut mucosa and gut-associated lymphoid tissue (Gowans and Knight, 1964; Griscelli *et al.*, 1969; Hall *et al.*, 1979; McDermott and Bienenstock, 1979; Rose *et al.*, 1976). Small lymphocytes from the efferent intestinal lymph also relocalize to gut-associated lymphoid tissues and efferent intestinal lymph (Chin and Hay, 1980; Scollay *et al.*, 1976). Several studies also suggest the existence of a lung-homing population of lymphoblasts and small lymphocytes (McDermott and Bienenstock, 1979; Spencer and Hall, 1984). Tissue-selective homing properties may enhance the efficiency of the immune system by targeting immune surveillance to tissues similar to those where antigen first entered the body; they may also reduce opportunity for autoimmune cross-reactions with self-antigens from other tissues (Picker and Butcher, 1992).

Tissue-selectivity is regulated, at least in part, by selective lymphocyte-endothelial cell recognition. Homing receptors are expressed on specific subpopulations of T cells, and recognise vascular addressins expressed on the endothelium. Recent work suggests that the local microenvironment at the time of lymphocyte activation in lymphoid tissue influences the expression of tissue-selective homing receptors (Picker *et al.*, 1993a; Picker *et al.*, 1993b). L-selectin has been identified as a homing receptor for PLN-homing cells, and the $\alpha 4\beta 7$ integrin LPAM-1 for mucosal homing cells. The former binds a 50 kDa glycoprotein expressed on PLN HEV endothelial cells, while the most likely ligand for LPAM-1 is a 55-65 kDa glycoprotein specifically expressed by HEVs in Peyer's patches and mesenteric lymph nodes (reviewed by Jutila (1994)). Homing mechanisms for migration of lymphocytes into inflamed skin and inflamed synovium have also been described. E-selectin acts as a vascular addressin on the endothelium of chronically inflamed skin, and binds a skin-associated subset of memory/effector T cells defined by expression of the cutaneous lymphocyte-associated (CLA) antigen (Picker *et al.*, 1991), which acts as a homing receptor (Berg *et al.*, 1991). T cell entry into the inflamed synovium may also involve a synovial vascular addressin (Jalkanen *et al.*, 1986; Salmi and Jalkanen, 1992).

1.2.2 *Inflammation*

Inflammation generally occurs as a defensive response to injury, and is commonly initiated by microbial infection, as well as mechanical trauma and toxins. It is a localised reaction which seeks to eliminate the inciting agent and restore the tissue to a normal state (Gallin *et al.*, 1992). Most forms of inflammation involve both humoral and cellular components of the immune system, and antigen may be recognised as "foreign" by specific or non-specific means. Non-specific recognition (ie. recognition of carbohydrates, denatured proteins or endotoxins) can be mediated by tissue phagocytes or the alternative complement pathway. Tissue macrophages respond by phagocytosis of the offending material and release of inflammatory cytokines such as IL-1, TNF and IL-8 (Muller-Eberhard, 1989; Wright, 1992). Microbes which activate the alternative complement pathway are opsonized with C3b, which enhances phagocytosis, and leads to formation of the membrane attack complex; in the process, the pro-inflammatory fragments C3a, C4a and C5a are released (Abbas *et al.*, 1991; Wright, 1992). Specific recognition is mediated by antigen-specific immunoglobulins or T cell receptors. The former activate the complement cascade, mainly by the classical pathway (Feinstein *et al.*, 1986). Complement fragments, C5a in particular, attract granulocytes and mononuclear phagocytes to the site of antibody deposition. These recognize antibody-coated target cells via Fc or complement receptors, leading to phagocytosis (Wright, 1992). T cell recognition of antigen, presented by tissue macrophages in association with MHC molecules, may induce a cell-mediated response, in which circulating phagocytic cells (neutrophils and monocytes) are induced to enter the infected tissue by T cell-derived cytokines such as IL-1 and TNF (Abbas *et al.*, 1991).

Both modes of recognition initiate the release of proinflammatory substances, such as cytokines, prostaglandins and leukotrienes released by cells participating in inflammatory processes (Baggiolini *et al.*, 1992; Davies and MacIntyre, 1992; Dinarello, 1992; Lam and Austen, 1992), or complement-derived peptides (Abbas *et al.*, 1991). These mediators alter blood flow, increase vascular permeability, activate vascular endothelium, and promote the migration of circulating neutrophils and monocytes into tissues. These phagocytic cells mediate the actual destruction of antigens (Gallin *et al.*, 1992).

The infiltration of leukocytes into extravascular tissue, and their subsequent activation, are central events in most forms of inflammation (Gallin *et al.*, 1992). The cellular infiltrate is composed of neutrophils and/or mononuclear cells,

depending on the nature of the inflammatory stimulus and the age of the lesion (Beekhuizen and van Furth, 1993; Issekutz *et al.*, 1981). Neutrophils generally predominate initially, due to a large but short-lived influx which peaks within in a few hours of the onset of inflammation. As the life-span of neutrophils within the tissue is short, monocytes, which enter more slowly, come to predominate at later stages. Lymphocytes are usually a minor component of the acute inflammatory exudate (van Furth *et al.*, 1979; Issekutz and Movat, 1980; Issekutz *et al.*, 1981; Paz and Spector, 1962). Chronic inflammation, which may derive from an unresolved acute inflammatory reaction

is also characterized by leukocyte infiltration of extravascular tissue. The cellular infiltrate consists mainly of mononuclear phagocytes, lymphocytes and plasma cells, although neutrophils and eosinophils may also be present (Gallin *et al.*, 1992; MacSween and Whaley, 1992).

1.2.3 Leukocyte migration into non-inflamed , non-lymphoid tissues

The bulk of leukocyte emigration into extravascular tissues occurs in the lymphoid tissues and at sites of inflammation. There is, however, some movement of blood monocytes into non-lymphoid tissues under steady-state conditions (Beekhuizen and van Furth, 1993). There they differentiate into either free- or fixed-tissue macrophages (MacSween and Whaley, 1992), and act as phagocytic scavengers (Johnston, 1988). Free-tissue macrophages migrate through the loose connective tissues of the peritoneum, lymphoid tissues, pulmonary alveoli and brain (microglia), while the fixed-tissue macrophages remain within a single organ, such as the sinusoids of the liver (Kupffer cells), spleen and bone marrow, and the lymphatic sinuses of lymph nodes (MacSween and Whaley, 1992).

Entry of lymphocytes into normal, non-lymphoid tissues occurs at a low level, demonstrated by the small amount of radioactivity found in tissues such as kidney, muscle, brain and skin after intravenous injection of labelled thoracic duct lymphocytes (Rannie and Donald, 1977), and the correspondingly low numbers of lymphocytes found in the afferent lymph draining all tissues except liver and gut mucosa. There are, for example, 200-800 lymphocytes/mm^3 in the afferent lymph draining skin or muscle, compared with is 2, 000-4, 000/mm^3 in efferent lymph or blood (Parrott and Wilkinson, 1981). Migration into non-lymphoid tissues is largely restricted to small lymphocytes with effector/memory phenotype, and lymphoblasts. Lymphocytes of the effector/memory phenotype have been isolated from the afferent lymph in sheep (Mackay *et al.*, 1990; Smith *et al.*, 1970). Blast cells isolated from *in vivo*

stimulated lymphoid tissues and injected into syngeneic recipients distribute between the lung, liver, spleen and skin in the case of PLN-derived cells, or enter the lamina propria of the gut in the case of blast cells derived from mesenteric lymph nodes. Lymphocytes activated *in vitro* by lectins and other mitogens behave similarly (reviewed by Hamann and Rebstock, 1993), and antigen-stimulated T cell blasts have been observed to enter the central nervous system (Hickey *et al.*, 1991; Ludowyk *et al.*, 1992). These cells subsequently return to the draining lymph nodes via the afferent lymphatics, and eventually to the blood via the efferent lymph. Constitutive migration of effector/memory lymphocytes and blast cells into non-lymphoid tissues may constitute an immune surveillance mechanism, whereby antigen-primed lymphocytes are able to respond promptly to recall antigens as soon as they enter the body (Mackay, 1991).

1.3 The barrier to leukocyte migration: structure of the venular wall

Entry of circulating leukocytes into extravascular tissues requires penetration of the vascular wall. In general, leukocyte extravasation is believed to occur in the postcapillary venular segments of the microvasculature (Marchesi, 1961). Post-capillary venules are similar in structure to capillaries, although larger, consisting of a single layer of endothelial cells overlying a thin, continuous basement membrane, and encased by a periendothelial sheath of pericytes, collagen fibres and a few elastic tissue fibres (Marchesi and Florey, 1960; Simionescu *et al.*, 1975).

Specialized post-capillary venules support the migration of lymphocytes into lymph nodes and the MALT of the gut and lungs. These are known as high-endothelial venules (HEVs) due to their cuboidal, plump cell morphology (Anderson *et al.*, 1976; reviewed by Kraal *et al.*, 1987). HEVs are a permanent feature of lymphoid tissues, however their number and length vary with local immune activity (Anderson *et al.*, 1975; Hendriks *et al.*, 1987), and HEV-like structures often develop at sites of chronic inflammation (Jalkanen *et al.*, 1986; Oppenheimer-Marks and Ziff, 1986). HEV morphology varies between species; endothelial cells in rats and guinea pigs are less cuboidal than those of mice and humans, while in sheep, HEVs are not discernable at all (Kraal *et al.*, 1987). In the rat, HEVs are lined by polygonal endothelial cells, separated by intercellular gaps of 200-400 Å, and joined by tight junctions near their apical and basal surfaces. This endothelium rests on a basement membrane. A complex sheath of connective tissue surrounds the venule, consisting of two or

three layers of overlapping reticular plates which are also covered by basement membrane, and are linked to the underlying stroma of the lymph node by collagen fibres (Anderson *et al.*, 1976; Wenk *et al.*, 1974).

1.3.1 Endothelial cells

Endothelial cells line all the blood vessels of the body, forming an interface between blood and tissues. In most tissues, they form a continuous monolayer of tightly connected cells, exceptions being highly specialized endothelium in liver, spleen and bone marrow, where the monolayer is discontinuous (Smith *et al.*, 1989). Morphologically, endothelial cells are relatively homogeneous. They are flattened, polygonal, elongated cells, approximately 25-50 µm long, 10-15 µm wide, and 3 µm thick near the nucleus, with the long axis oriented in the direction of the bloodflow. Regional differences include some variation in size and thickness, and in relative content of organelles and vesicles (reviewed by Thorgeirsson and Robertson, 1978).

Endothelial cells are connected by tight junctions, which contribute to their barrier function, and in some cases are also connected by gap junctions, which permit communication between cells (Alberts *et al.*, 1986; Huttner *et al.*, 1973; Simionescu *et al.*, 1975). Each segment of the circulation has a characteristic arrangement of intercellular junctions. Arterial and arteriolar endothelial cells are joined by a composite system of tight junctions and gap junctions, while capillaries and post-capillary venules have tight junctions only (Simionescu *et al.*, 1975; Simionescu *et al.*, 1976). Post-capillary venules have the least organised endothelial junctions (Simionescu *et al.*, 1975), which may be a factor in their being the favoured entry point for leukocytes during inflammation. Tight junctions create a diffusion barrier in the plasma membrane (Alberts *et al.*, 1986), and the lumenal and ablumenal membranes of endothelial cells are chemically and functionally distinct. The lumenal membrane is non-adhesive under normal conditions, allowing the free flow of blood cells, while the ablumenal membrane firmly adheres to the basement membrane, anchoring the cell to the subendothelial extracellular matrix (Defilippi *et al.*, 1993).

1.3.2 The extracellular matrix and basement membranes

The extracellular matrix (ECM) is an intricate network of macromolecules which fills the extracellular space in tissues, and includes the basement membranes and connective tissue stromas. At the molecular level, it consists largely of proteoglycans which form a highly hydrated, gel-like framework in

which structural (collagen and elastin) and adhesive (fibronectin and laminin) fibrous proteins are embedded. The proteoglycan gel permits the diffusion of nutrients, metabolites and hormones between capillaries and tissue cells. Collagen fibres strengthen and organise the matrix, elastin fibres provide resilience, and adhesive proteins mediate cell attachment (Alberts *et al.*, 1986; Shimizu, 1991).

Basement membranes are specialized extracellular matrices found in most multicellular animal species. They are produced by epithelial cells, endothelial cells and many mesenchymal cells, forming a thin, but tough, sheet-like structure at the interface of cell layers and the underlying connective tissue (Fig. 1.1) (Vracko, 1974; Yurchenco and Schnittny, 1990). Basement membranes also completely surround adipocytes, muscle cells and Schwann cells (Abrahamson, 1986), as well as some organs such as the kidney (Alberts *et al.*, 1986). The basement membrane provides physical support for cells and cell layers (Vracko, 1974); acts as a selective molecular sieve between tissue compartments, which impedes the passage of cells and macromolecules; and functions in the regulation of cell growth, attachment and differentiation (Yurchenco and Schnittny, 1990). Most basement membranes have at least three morphologically distinct layers, discernible by electron microscopy. The lamina lucida, approximately 40-60 nm in thickness, lies directly beneath the plasma membrane of the overlying cell layer. The central lamina densa, or basal lamina, is an electron dense layer of similar thickness. The outer layer is known as the lamina fibroreticularis or sub-lamina densa. Fine strands of fibrillar material connect the basal lamina and overlying cell membranes, and in some cases anchor also it to the underlying connective tissue (Abrahamson, 1986).

Although the precise composition of the basement membrane varies between tissues, all contain type IV collagen, the glycoproteins laminin and entactin, and proteoglycans (Alberts *et al.*, 1986; Schultz, 1991):

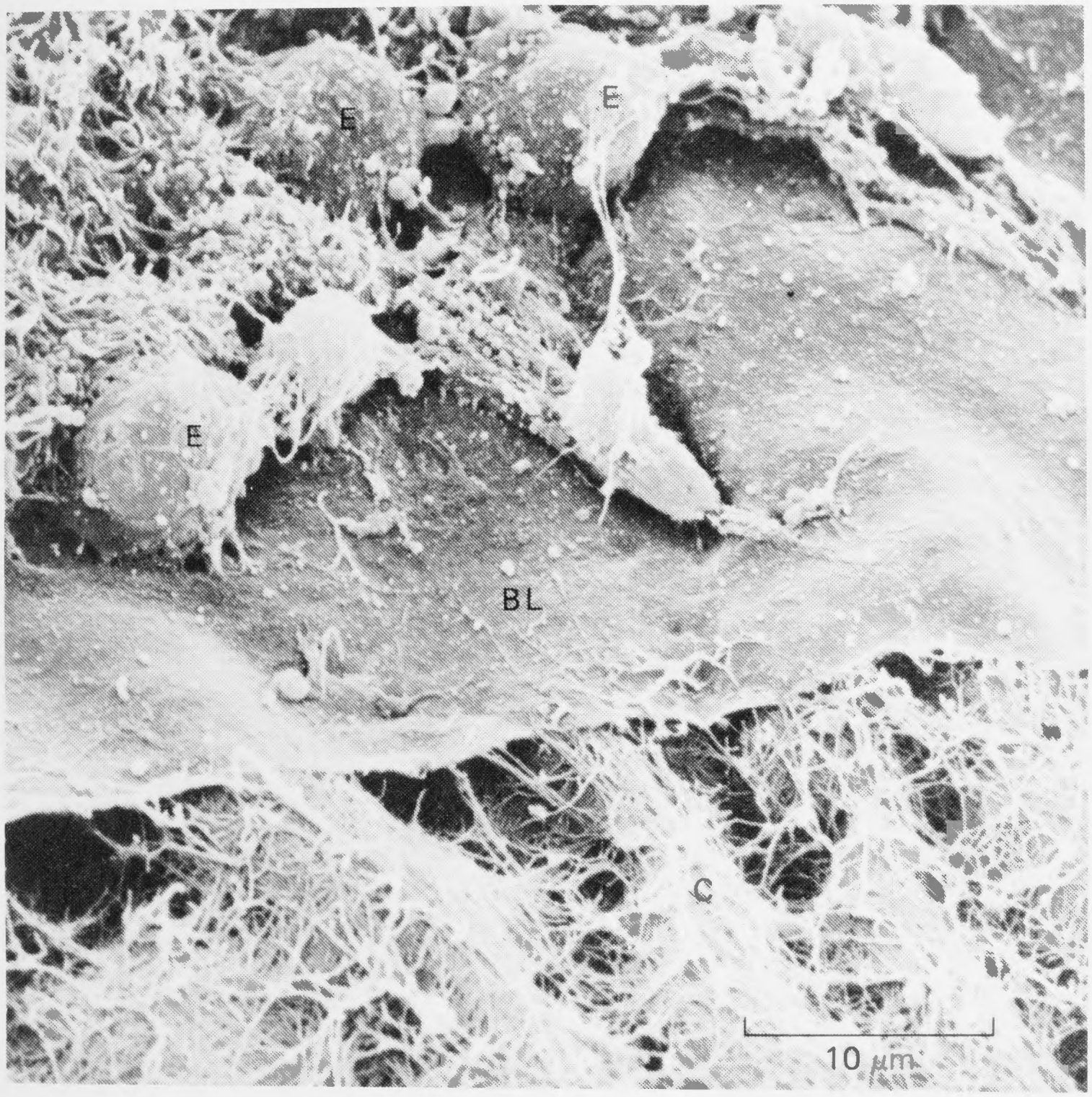
Type IV collagen is unique to basement membranes, and unlike other ECM collagens (types I-III), does not form fibrils. Instead rod-like monomers associate by their C-terminal domains to form head-to-head dimers, while tetramers form by lateral association of N-terminal regions (Fig. 1.2) (Mignatti and Rifkin, 1993; Timpl, 1989).

Laminin is a flexible, cross-shaped glycoprotein, consisting of three polypeptide chains held together by disulfide bridges (Fig. 1.2). It has binding sites for

Fig. 1.1

Scanning electron micrograph of the basement membrane in the cornea of a chick embryo. Epithelial cells (E) have been removed to expose the upper surface of the basement membrane (BL). A network of collagen fibrils (C) interacts with the lower surface.

Photograph from Alberts *et al.*, (1986).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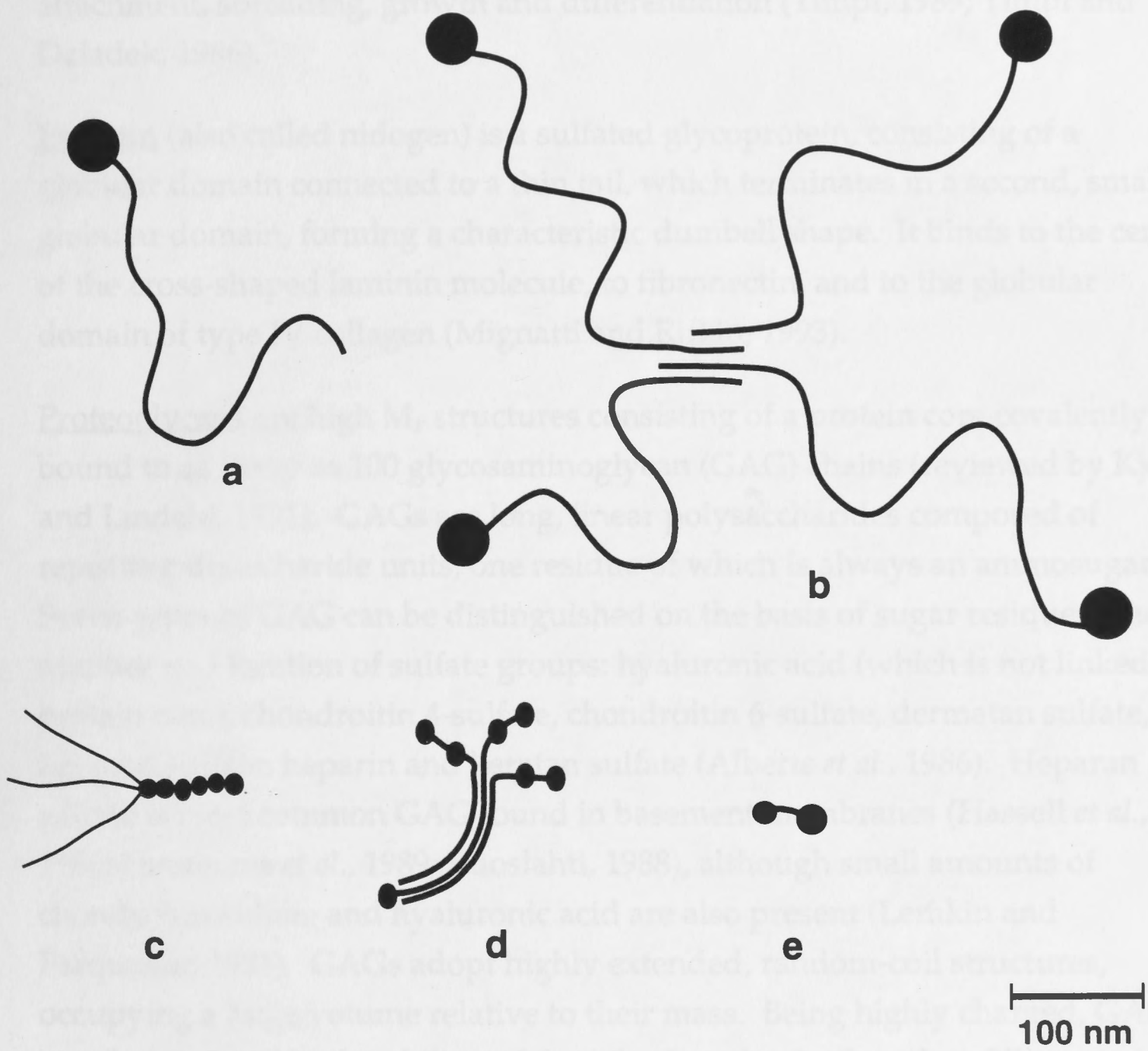


Fig. 1.2 Scale diagrams of basement membrane macromolecules.

a. Type IV collagen monomers form a triple-stranded helical rod, terminating in a globular C-terminal domain.
b. Type IV collagen tetramers form by lateral association between N-terminal segments arranged alternately in parallel and antiparallel fashion. Networks of collagen IV may also form by crosslinking of tetramers at the globular C-terminal domains and by lateral associations along the arms.
c. Heparan sulfate proteoglycan showing three heparan sulfate chains bound to a smaller core protein. Larger forms also exist.
d. Laminin
e. Entactin

Adapted from Abrahamson (1986), Timpl (1989), Yurchenko and Schittny (1990)

interstitial and type IV collagens, heparan sulfate, entactin, and cell surface laminin receptors. The latter mediate cell-matrix interactions, and influence cell attachment, spreading, growth and differentiation (Timpl, 1989; Timpl and Dziadek, 1986).

Entactin (also called nidogen) is a sulfated glycoprotein, consisting of a globular domain connected to a thin tail, which terminates in a second, smaller globular domain, forming a characteristic dumbell shape. It binds to the centre of the cross-shaped laminin molecule, to fibronectin, and to the globular domain of type IV collagen (Mignatti and Rifkin, 1993).

Proteoglycans are high M_r structures consisting of a protein core covalently bound to as many as 100 glycosaminoglycan (GAG) chains (reviewed by Kjellin and Lindahl, 1991). GAGs are long, linear polysaccharides composed of repeating disaccharide units, one residue of which is always an aminosugar. Seven types of GAG can be distinguished on the basis of sugar residues, and number and location of sulfate groups: hyaluronic acid (which is not linked to a protein core), chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparan sulfate, heparin and keratan sulfate (Alberts *et al.*, 1986). Heparan sulfate is most common GAG found in basement membranes (Hassell *et al.*, 1986; Heremans *et al.*, 1989; Ruoslahti, 1988), although small amounts of chondroitin sulfate and hyaluronic acid are also present (Lemkin and Farquahar, 1981). GAGs adopt highly extended, random-coil structures, occupying a huge volume relative to their mass. Being highly charged, GAGs are also strongly hydrophilic and form hydrated gels, thus they fill most of the extracellular space and provide mechanical support to tissues (Alberts *et al.* 1986; Mignatti and Rifkin, 1993).

According to current models, the basement membrane is a polygonal meshwork of polymerized type IV collagen and laminin, bridged by entactin and associated with heparan sulfate proteoglycans (Ratner, 1992; Schultz, 1991; Yurchenco and Schnittny, 1990). A schematic representation of the three dimensional structure of the three major components, collagen type IV, laminin and heparan sulfate proteoglycan (HSPG), is depicted in Fig. 1.3. Type IV collagen provides mechanical suppport by forming a sheet-like, polygonal framework through covalently stabilized N- and C-terminal and lateral associations. Laminin self-associates through terminal domain interactions to form a second lattice within the collagen framework. These two networks are connected by entactin, whose C-terminal domain binds both laminin and type IV collagen. Proteoglycans are firmly anchored in the basement membrane; the

Fig. 1.3

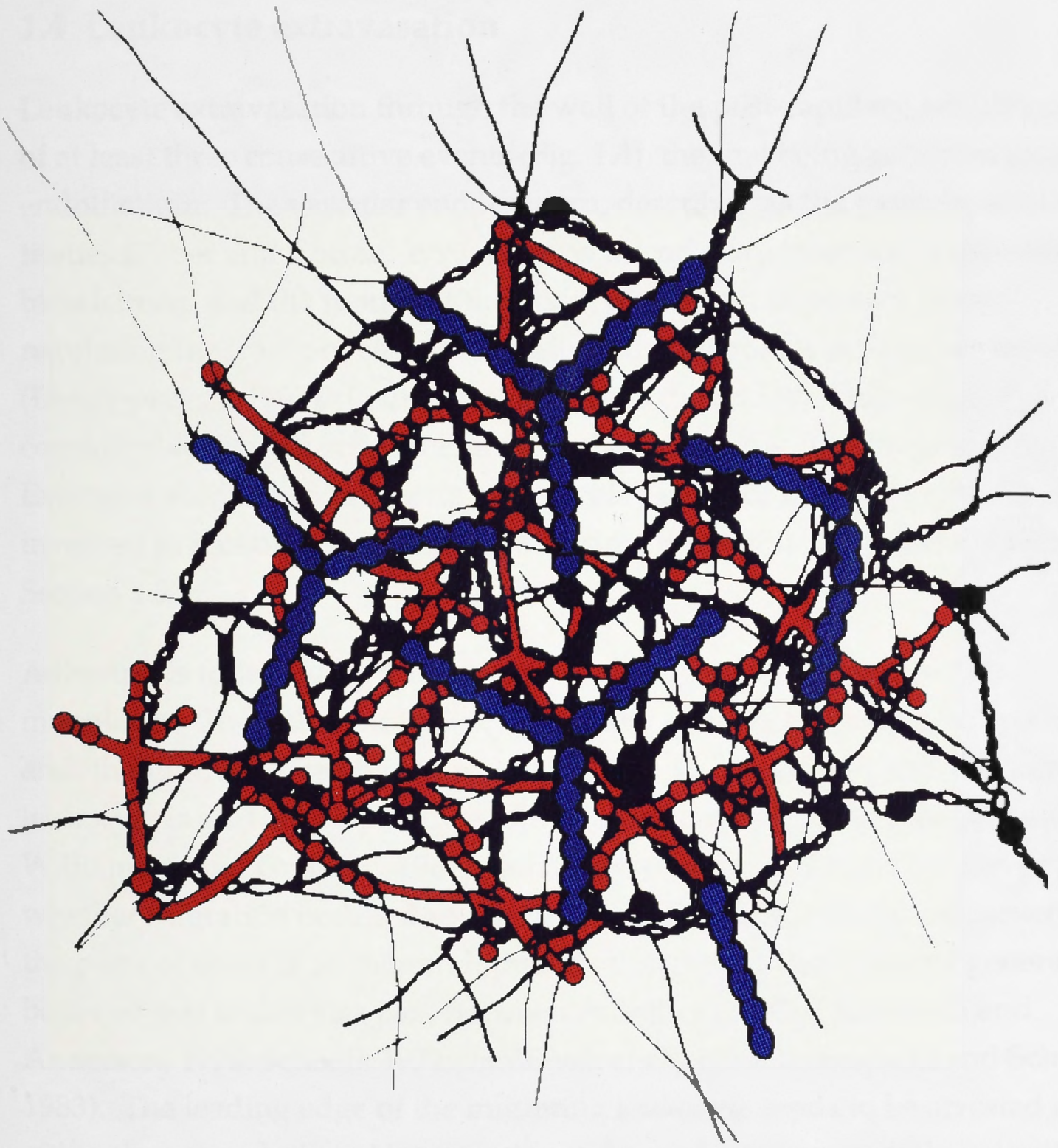
Schematic representation of the three dimensional structure of the three major components of the basement membrane: type IV collagen (black), laminin (red) and heparan sulfate proteoglycan (blue). Type IV collagen and laminin form double polymer networks. Heparan sulfate proteoglycan complexes interact with collagen and laminin through their polyanionic sidechains.

Adapted from Yurchenco and Schittney (1990).

Collagen type IV

Laminin

Heparan sulfate proteoglycan



protein cores bind one another to form oligomers, while GAG chains bind weakly to laminin and type IV collagen (Yurchenco and Schnittny, 1990). The high negative charge of sulfated GAG chains is important in controlling basement membrane permeability to charged macromolecules (Kanwar, 1984), and also has a charge-sieving effect, as these polyanionic chains possess large spheres of hydration which leave very little free space in the basement membrane, allowing passage to small macromolecules only (Yurchenco and Schnittny, 1990).

1.4 Leukocyte extravasation

Leukocyte extravasation through the wall of the post-capillary venule consists of at least three consecutive events (Fig. 1.4), the first being adhesion to the endothelium. The vascular endothelium, described as the gatekeeper of the tissues (Pober and Cotran, 1990a), forms the primary interface between the bloodstream and the tissues of the body, playing an important role in regulating the traffic of circulating cells and metabolites across the vessel wall (Defilippi *et al.*, 1993). Leukocytes adhere to the endothelium despite considerable shear forces generated by the blood flow (Luscinskas *et al.*, 1994). Extensive study in recent years has revealed some of the molecular mechanisms involved in leukocyte-endothelial adhesion; these will be further discussed in Section 1.5.1.

Adhesion is followed by transmigration through the endothelial cell monolayer. The adherent leukocyte initially extends a pseudopod into the endothelium, followed by the rest of the cell. In some cases, the migrating leukocyte is also enveloped by endothelial cytoplasmic extensions (Shaw, 1980; Williamson and Grisham, 1961). It has been a matter of some controversy as to whether migration occurs through the body of the endothelial cell, or whether the point of entry is an interendothelial cell junction, but it is now generally believed that leukocytes pass between endothelial cells (Anderson and Anderson, 1976; Schoefl, 1972; van Ewijk *et al.*, 1975; Yamaguchi and Schoefl, 1983). The leading edge of the migrating leukocyte tends to be arrested at either the subendothelial basement membrane or the periendothelial cell layer until most or all of the leukocyte has passed beneath the endothelium, after which any defect in the endothelial layer is sealed (Hurley and Xeros, 1961; Schoefl, 1972; Shaw, 1980). The barrier function of the endothelium is preserved during transmigration, due to close interaction between the peripheral membranes of migrating leukocytes and endothelial cells, although

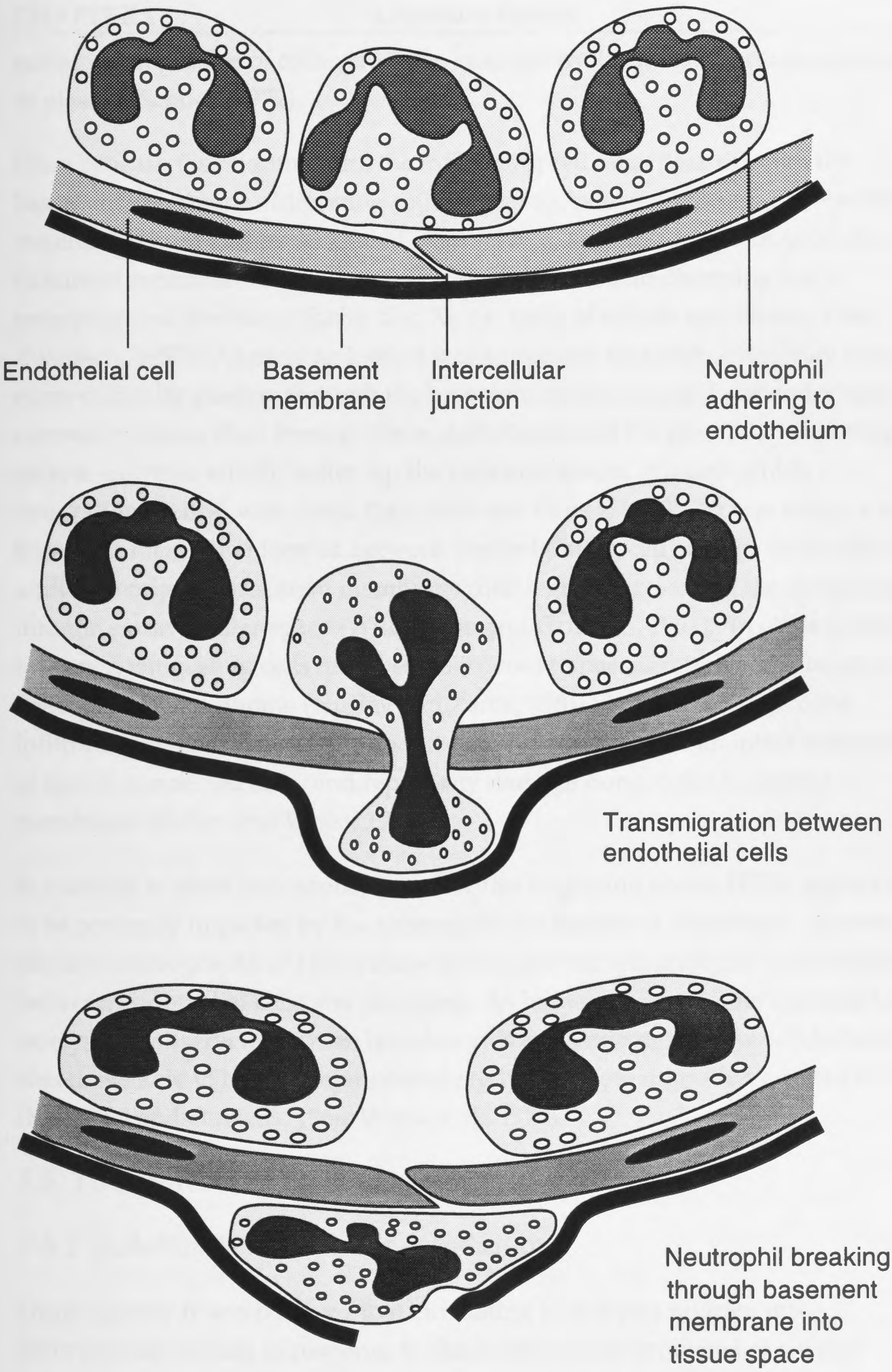


Fig. 1.4

Stages in the extravasation of neutrophils (or other leukocytes) through the vascular wall. Adapted from Bartlett (1993).

sustained migration of cells across the vascular wall can result in some leakage of plasma (Schoefl, 1972).

Once beneath the endothelium, the infiltrating cell must pass through the basement membrane. Migrating cells appear to "pause" in the space between the endothelium and periendothelial structures, both inside and outside the basement membrane, with the pressure of the leukocyte distorting the overlying endothelium (Hurley and Xeros, 1961; Marchesi and Florey, 1960; Zweifach, 1955). As early as 1960, it was suggested that leukocytes may have more difficulty passing through the basement membrane and periendothelial connective tissue than through the endothelium, and the possibility that they secrete enzymes which "soften up the cells and tissues through which emigration occurs" was raised (Marchesi and Florey, 1960). In one study, a new basement membrane formed between the emigrating cell and the endothelium while the original basement membrane disintegrated, releasing the lymphocyte into the extravascular space (Williamson and Grisham, 1961). In other studies, however, emigrating cells have been observed to pass through small holes in the basement membrane (Hurley and Xeros, 1961; Shaw, 1980). Once the infiltrating cell has passed, the endothelial cells reconstruct an intact monolayer of tightly connected cells, and repair any damage done to the basement membrane (Huber and Weiss, 1989).

In contrast to other endothelia, lymphocytes migrating across HEVs appear not to be seriously impeded by the subendothelial basement membrane. Scanning electron micrographs of HEVs show that migrating lymphocytes accumulate between endothelial cells and pericytes. As basement membrane material has rarely been observed between lymphocytes and pericytes, the periendothelial sheath is likely to be the major obstacle to lymphocyte migration across HEVs (Marchesi and Gowans, 1964; Wenk *et al.*, 1974).

1.5 The molecular basis of extravasation

1.5.1 Adhesion to the vascular endothelium

Until recently it was believed that circulating leukocytes migrate into extravascular tissues in response to chemoattractants produced at sites of inflammation (Goldstein *et al.*, 1977). Leukocytes were thought to "sense" the presence of a pathogen via specific chemoattractant receptors: once exposed to the chemoattractant within the vasculature, leukocytes were activated, becoming more adhesive for endothelium, and followed the "trail" of the

chemoattractant into the extravascular space. The role of the endothelium in this model was one of passive retraction, permitting the leukocytes to pass through (Cronstein and Weissman, 1993).

Recent work has shown that the endothelium actually plays a very active role in leukocyte extravasation, expressing adhesion molecules specific for counter-receptors on circulating leukocytes. Diapedesis under non-inflammatory conditions is relatively restricted: recirculating lymphocytes enter lymph nodes by binding to and migrating across HEVs; blood monocytes leave the circulation at a low frequency to become tissue macrophages (van Furth, 1986); and lymphocytes also enter non-lymphoid, non-inflamed tissues at a low rate (Janossy *et al.*, 1989; Mackay *et al.*, 1990; Rannie and Donald, 1977). During inflammation, however, the endothelium lining the postcapillary venule is activated by cytokines and other mediators produced in the extravascular tissue, inducing or upregulating expression of adhesion molecules (Munro *et al.*, 1989). According to this revised model, circulating leukocytes, which constitutively display receptors for endothelial adhesion molecules, adhere transiently to the stimulated endothelium. Once bound, they receive an activation signal, delivered by cytokines or cell surface interactions, which enables them to adhere more tightly, and migrate through the endothelial monolayer. Once in the extravascular tissue, leukocytes follow a chemoattractant gradient toward the inciting pathogen (Butcher, 1991; Cronstein and Weissman, 1993; Osborn, 1990).

The adhesion molecules involved in leukocyte-endothelial interactions belong to three major classes: selectins, integrins and the immunoglobulin superfamily. A primary interaction between a selectin and its carbohydrate ligand is largely responsible for the initial binding of leukocytes to endothelium; this is a relatively unstable interaction and leads to leukocyte "rolling" along the vessel wall. Activation of adherent leukocytes leads to functional activation of β_1 and β_2 integrins, which adhere to members of the immunoglobulin superfamily on endothelial cells in an interaction which is stable under shear force, and leads to the complete arrest of leukocyte movement over the endothelium (Fig. 1.5). Integrin activation can be triggered by selectin-mediated binding (Lo *et al.*, 1991), and by a variety of chemoattractants and stimulants, such as IL-8 (Detmers *et al.*, 1990), PAF, C5a and endotoxin (Pober and Cotran, 1990b; Tonnesen *et al.*, 1989), that might be displayed on the endothelial surface. These adhesive interactions, and the properties of the selectins, integrins and immunoglobulin superfamily, have been extensively reviewed by Butcher

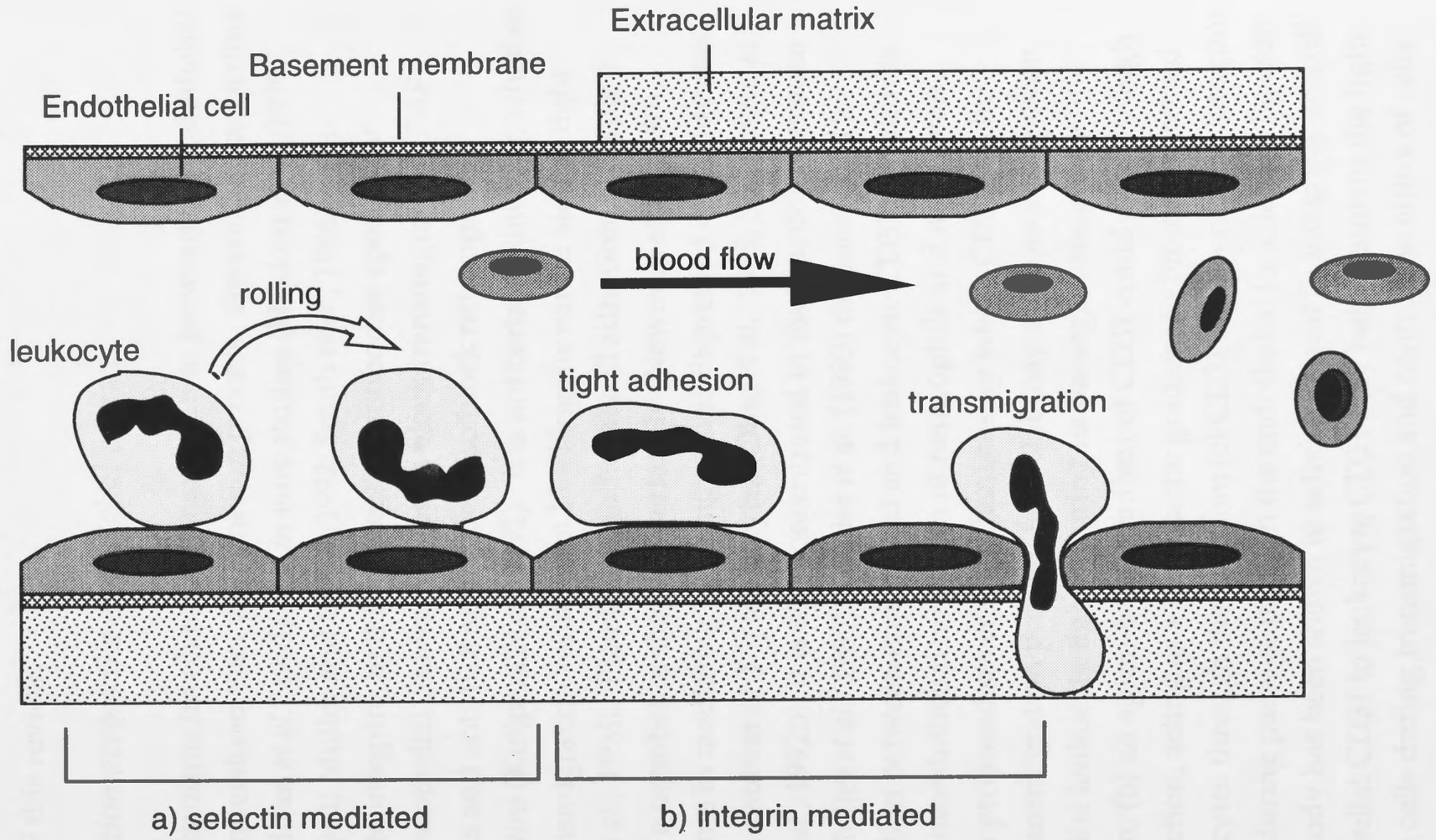


Fig. 1.5 Model for the adhesion mechanisms underlying the migration of circulating leukocytes into extravascular tissues.

a) Selectins initiate rolling contacts between leukocytes and endothelium, allowing time for the leukocytes to become exposed to locally released signaling molecules.

b) Integrins on the activated leukocytes mediate shear-resistant attachment and transendothelial migration

Adapted from Bartlett (1993), Springer (1990) and Shimizu *et al.* (1991).

(1991), Dustin and Springer (1991), Hogg (1991), Hogg (1992), Hynes (1992), Mackay (1993), Pardi *et al.* (1992), Pober and Cotran (1991b), Schweighhoffer and Shaw (1992), Shimizu *et al.* (1992), Sonnenberg (1993), Tanaka *et al.* (1993c), Vestweber (1993), and Zimmerman *et al.* (1992), and further discussion is beyond the scope of this review.

1.5.2 Transmigration across the endothelial barrier

The molecular mechanisms involved in transmigration between the endothelial cells are less well understood than the adhesion process. Several *in vivo* (Arfors *et al.*, 1987; von Andrian *et al.*, 1991) and *in vitro* studies (Hakkert *et al.*, 1990; Kavanaugh *et al.*, 1991; Muller and Weigl, 1992; Smith *et al.*, 1988) have implicated leukocyte integrins in this process, as antibodies specific for integrins or their endothelial counter-receptors block transmigration. PAF receptor antagonists and anti-IL 8 antibodies also block neutrophil transmigration *in vitro* (Kuijpers *et al.*, 1992). It is not certain, however, whether these effects on transmigration result from blocking the earlier step of tight adhesion (Muller *et al.*, 1993). Platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31), a member of the Ig superfamily (Newman *et al.*, 1990), appears to have a role in mediating transmigration. Expressed on monocytes, neutrophils and on a subset of T lymphocytes (Ohto *et al.*, 1985; Stockinger *et al.*, 1990; Tanaka *et al.*, 1992b), it is also concentrated in the junctions between endothelial cells (Muller *et al.*, 1989). Muller *et al.* (1993) demonstrated a homophilic interaction between endothelial and leukocyte CD31, which was required for the transendothelial migration of neutrophils and monocytes. These investigators proposed several mechanisms by which CD31 might contribute to the transmigration process: (a) CD31 may serve as an adhesion molecule, binding the leukocyte tightly during its passage through the endothelial junction; (b) an apical-basal gradient of CD31 could exist through the endothelial junction, acting as a haptotactic gradient to produce directed migration of leukocytes through the junction; (c) CD31 could enhance integrin-mediated binding during passage through the endothelial junction, as ligation of CD31 with antibody has been shown to activate β_1 and β_2 integrins; and (d) adhesion of endothelial CD31 to leukocyte CD31 may help maintain the tight apposition of these cells during transmigration and control the efflux of cells and soluble molecules.

1.5.3 Passage of leukocytes through the subendothelial basement membrane

Having passed through the endothelial lining of the vasculature, migrating leukocytes must then penetrate the subendothelial basement membrane. Most studies concerning leukocyte entry into tissues have concentrated on the adhesion step; few have considered the basement membrane as a serious barrier. Since it regulates the passage of plasma proteins and other macromolecules from the capillaries into tissues (Vracko, 1974), it is reasonable to suppose that it also obstructs the path of extravasating leukocytes. This has long been recognised in the field of tumour metastasis, where mechanisms involved in the degradation of basement membranes and interstitial stroma by invading tumour cells have been extensively studied.

According to current models of extravasation (Fig. 1.6) (Liotta, 1986; Ratner, 1992), the invading cell attaches to the ECM via specific adhesion molecules, which bind reversibly to matrix components such as laminin (in the basement membrane) or fibronectin (in the connective tissue stroma). Once anchored, the cell secretes (or induces host cells to secrete) proteolytic and glycolytic enzymes. These appear to play a vital role by clearing a path for the cell through the dense meshwork of the ECM, as well as by more subtle means, such as reversing adhesive contacts and possibly degrading ECM components into motility-stimulating fragments. The final step involves cell locomotion into the region of the matrix modified by hydrolysis. Continued movement of the cell may take place by cyclic repetition of these steps, implying that hydrolysis of ECM components must be tightly regulated in a temporal and spatial fashion with respect to cell attachment and migration. Insufficient degradation would prevent forward movement, while excessive degradation would result in a loss of the substratum needed for cell attachment to the ECM (Stetler-Stevenson *et al.*, 1993).

1.6 Hydrolytic enzymes and basement membrane degradation

The role of hydrolytic enzymes in ECM degradation has been studied most comprehensively in the context of tumour growth and metastasis. Once cancer cells detach and escape from the primary tumour, they move through connective tissue stroma and basement membranes at many key points in the metastatic cascade, for example during entry or exit from blood vessels, the invasion of muscle or nerve, and in traversing epithelial boundaries (Liotta, 1986). A number of studies have shown that metastatic tumour cells have the

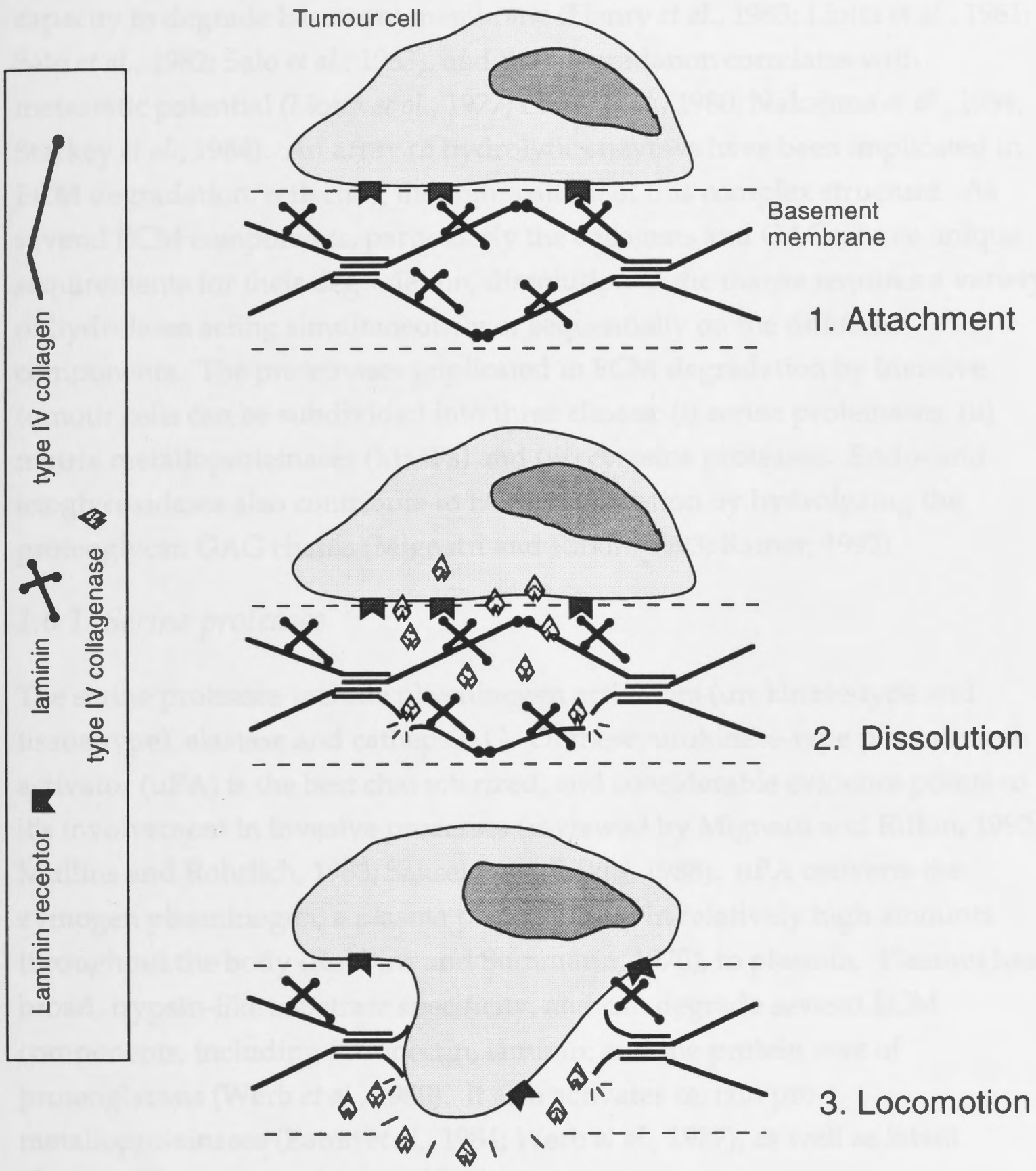


Fig. 1.6 Three step model for tumour cell invasion of the extracellular matrix.

Schematic diagram (not to scale) showing invasion of the basement membrane.
1. Tumour cell attachment to matrix components, such as laminin.
2. Local degradation of the matrix by tumour cell-associated proteinases. These may degrade attachment proteins as well as the structural proteins of the matrix.
3. Tumour cell locomotion into the region of the membrane modified by proteolysis. Continued invasion of the extracellular matrix may take place by cyclic repetition of these three steps.

Adapted from Liotta (1986).

capacity to degrade basement membrane (Henry *et al.*, 1983; Liotta *et al.*, 1981; Salo *et al.*, 1982; Salo *et al.*, 1983), and that degradation correlates with metastatic potential (Liotta *et al.*, 1977; Liotta *et al.*, 1980; Nakajima *et al.*, 1984; Starkey *et al.*, 1984). An array of hydrolytic enzymes have been implicated in ECM degradation, reflecting the composition of this complex structure. As several ECM components, particularly the collagens and GAGs, have unique requirements for their degradation, dissolution of the matrix requires a variety of hydrolases acting simultaneously or sequentially on the different components. The proteinases implicated in ECM degradation by invasive tumour cells can be subdivided into three classes: (i) serine proteinases, (ii) matrix metalloproteinases (MMPs) and (iii) cysteine proteases. Endo- and exoglycosidases also contribute to ECM degradation by hydrolyzing the proteoglycan GAG chains (Mignatti and Rifkin, 1993; Ratner, 1992).

1.6.1 Serine proteases

The serine proteases include plasminogen activators (urokinase-type and tissue-type), elastase and cathepsin G. Of these, urokinase-type plasminogen activator (uPA) is the best characterized, and considerable evidence points to it's involvement in invasive processes (reviewed by Mignatti and Rifkin, 1993; Mullins and Rohrlich, 1983; Saksela and Rifkin, 1988). uPA converts the zymogen plasminogen, a plasma protein found in relatively high amounts throughout the body (Robbins and Summaria, 1970), to plasmin. Plasmin has a broad, trypsin-like substrate specificity, and can degrade several ECM components, including fibronectin, laminin, and the protein core of proteoglycans (Werb *et al.*, 1980). It also activates certain pro-metalloproteinases (Eaton *et al.*, 1984; Werb *et al.*, 1977), as well as latent elastase (Chapman and Stone, 1984).

uPA is secreted as a proenzyme (pro-uPA) which binds to a specific cell surface receptor (uPA-R), a heavily glycosylated protein linked to the plasma membrane by a glycosyl-phosphatidylinositol anchor (Estreicher *et al.*, 1989; Roldan *et al.*, 1990). Receptor-bound pro-uPA is converted to its active form by limited proteolysis (Dano *et al.*, 1985; Petersen *et al.*, 1988). This can be accomplished by trace amounts of plasmin, and probably other proteinases, as uPA is activated *in vitro* by a membrane-bound serine proteinase on transformed chicken fibroblasts (Berkenpas and Quigley, 1991), and by the cysteine proteinase cathepsin B (Kobayashi *et al.*, 1991). As active uPA increases plasmin levels, the production of uPA generates a self-maintained feedback loop of pro-uPA and plasminogen activation (Fig. 1.7). The levels of

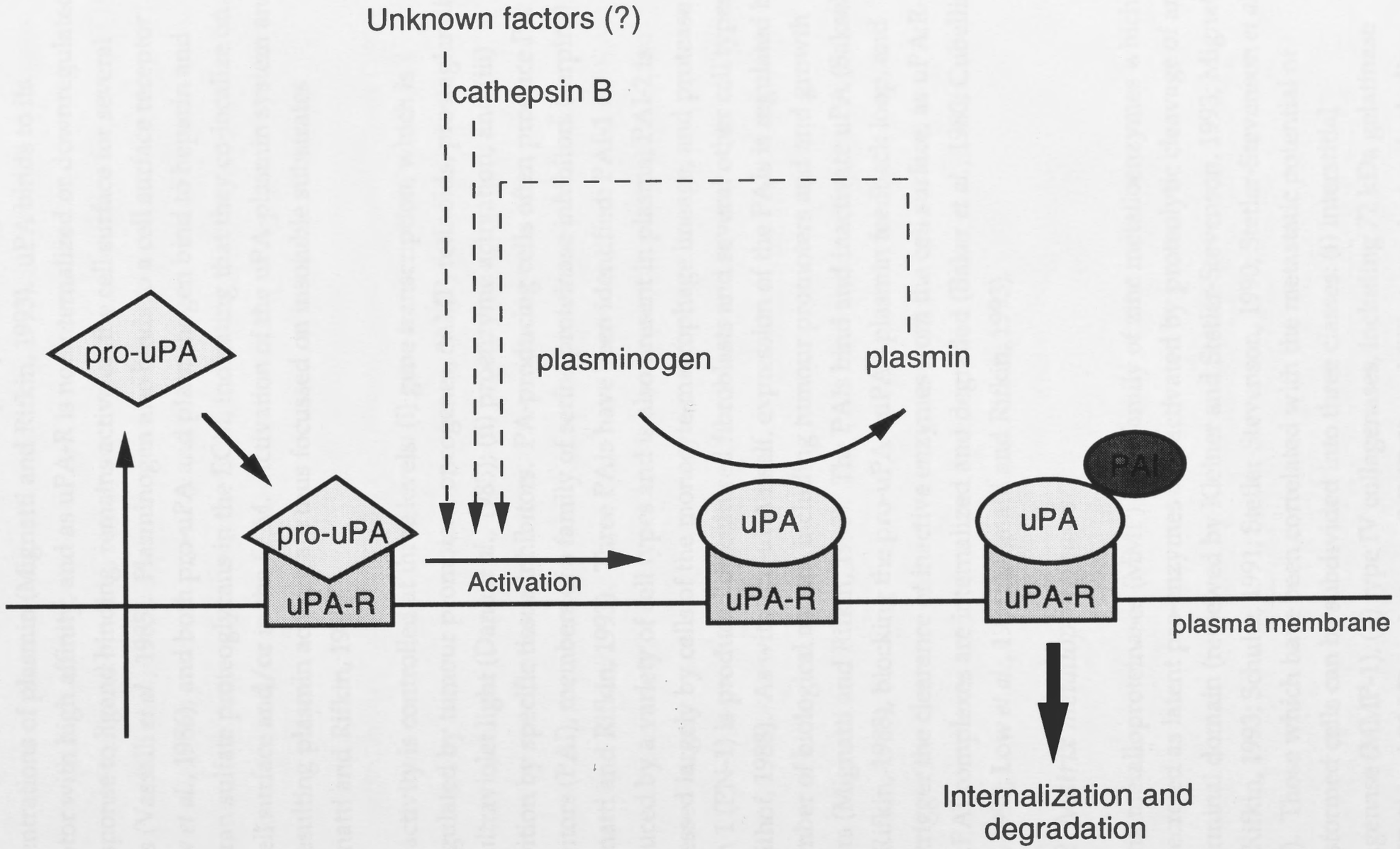


Fig. 1.7 Activation of urokinase-type plasminogen activator.

Abbreviations: uPA (urokinase-type plasminogen activator), uPA-R (uPA receptor), PAI (plasminogen activator inhibitor), – – – ► activation.

Adapted from Mignatti and Rifkin (1993).

plasminogen in the extravascular space are quite high, hence the initial activation of a small amount of uPA can rapidly lead to high local concentrations of plasmin (Mignatti and Rifkin, 1993). uPA binds to its receptor with high affinity, and as uPA-R is not internalized or downregulated in response to ligand binding, remains active on the cell surface for several hours (Vassalli *et al.*, 1985). Plasminogen also binds to a cell surface receptor (Plow *et al.*, 1986), and both pro-uPA and plasminogen bind to heparin and heparan sulfate proteoglycans in the ECM, indicating that they co-localize on the cell surface and/or in the ECM. Activation of the uPA-plasmin system and the resulting plasmin activity are thus focussed on insoluble substrates (Mignatti and Rifkin, 1993).

uPA activity is controlled at three levels: (i) gene transcription, which is upregulated by tumour promoters, oncogenes, cAMP, retinoids, prostaglandins and ultraviolet light (Dano *et al.*, 1985); (ii) proenzyme activation; and (iii) inhibition by specific tissue inhibitors. PA-producing cells often produce PA inhibitors (PAI), members of a family of serine proteinase inhibitors (serpins) (Mignatti and Rifkin, 1993). Three PAIs have been identified: PAI-1 is produced by a variety of cell types and is also present in plasma, PAI-2 is expressed largely by cells of the monocyte-macrophage lineage, and protease nexin 1 (PN-1) is produced by cultured fibroblasts and several other cell types (Kruithof, 1988). As with pro-uPA itself, expression of the PAIs is regulated by a number of biological agents, including tumour promoters and and growth factors (Mignatti and Rifkin, 1993). The PAIs bind and inactivate uPA (Saksela and Rifkin, 1988), blocking the pro-uPA - uPA - plasmin feedback loop, and also trigger the clearance of inactive enzymes from the cell surface, as uPA:R-uPA:PAI complexes are internalized and degraded (Baker *et al.*, 1980; Cubellis *et al.*, 1990; Low *et al.*, 1981; Mignatti and Rifkin, 1993).

1.6.2 Matrix metalloproteinases

Matrix metalloproteinases (MMP) are a family of zinc metalloenzymes which are secreted as latent pro-enzymes and activated by proteolytic cleavage of an N-terminal domain (reviewed by Kleiner and Stetler-Stevenson, 1993; Mignatti and Rifkin, 1993; Schultz, 1991; Stetler-Stevenson, 1990; Stetler-Stevenson *et al.*, 1993). Those which have been correlated with the metastatic potential of transformed cells can be subdivided into three classes: (i) interstitial collagenase (MMP-1), (ii) type IV collagenases, including 72 kDa gelatinase (MMP-2) and 92 kDa gelatinase (MMP-9), and (iii) stromelysins, including stromelysin-1 (MMP-3), stromelysin-2 (MMP-10) and matrilysin (MMP-7). The

substrate specificities of these metalloproteinases overlap somewhat. Interstitial collagenase degrades collagen types I-III, VII and X. The type IV collagenases degrade basement membrane (type IV) collagen, collagen types V, VII and X, gelatin and fibronectin, but not the interstitial collagens (types I-III). Stromelysins have the broadest substrate specificity, cleaving collagen types IV, V, VIII and IX, fibronectin, laminin, elastin, and the protein core of proteoglycans (Mignatti and Rifkin, 1993; Schultz, 1991). Between them, the MMPs are capable of degrading all the structural components of the ECM, with the exception of the proteoglycan GAG chains and hyaluronic acid (Woolley, 1993).

As with uPA, MMP activity is regulated at three levels: (i) gene transcription, (ii) pro-enzyme activation, and (iii) inhibition by specific tissue inhibitors. MMPs are not constitutively expressed, instead transcription is induced by such agents as tumour promoters, growth factors and oncogene products (reviewed by (Mignatti and Rifkin, 1993). The pro-enzyme form is activated by proteolytic cleavage of an amino terminal fragment (Stetler-Stevenson, 1990); *in vivo* this is accomplished for most of the MMP family by plasmin or other metalloproteinases. MMPs initially activated by plasmin can in turn activate other pro-enzyme molecules, creating an amplification loop similar to the feedback mechanism of pro-uPA and plasmin activation (Mignatti and Rifkin, 1993). Other activation mechanisms also exist, for example, the 72 kDa gelatinase appears to be activated by a poorly characterised cell surface receptor/activator system distinct from the uPA system (Kleiner and Stetler-Stevenson, 1993). Fully activated enzymes are regulated by binding of specific inhibitors, the TIMPs (tissue inhibitor of metalloproteinases), which appear to be members of a multigene family. Expression of these inhibitors is modulated by growth factors, hormones and cytokines. TIMP-1 and TIMP-2 bind non-covalently to active forms of all the MMPs, and to the latent forms of gelatinase A and B respectively, apparently controlling the activation of these two proenzymes (Kleiner and Stetler-Stevenson, 1993; Mignatti and Rifkin, 1993; Stetler-Stevenson, 1990).

Activation of MMPs and the uPA-plasmin system is thought to initiate a cascade of proteolytic events, which generates a broad spectrum of proteolytic activities (Figure 1.8). The uPA-plasmin system plays a key role in the proposed cascade. The activated form of uPA can rapidly convert the high concentrations of plasminogen found in most tissues to plasmin. Plasmin plays a dual role, directly degrading ECM components, as well as activating pro-

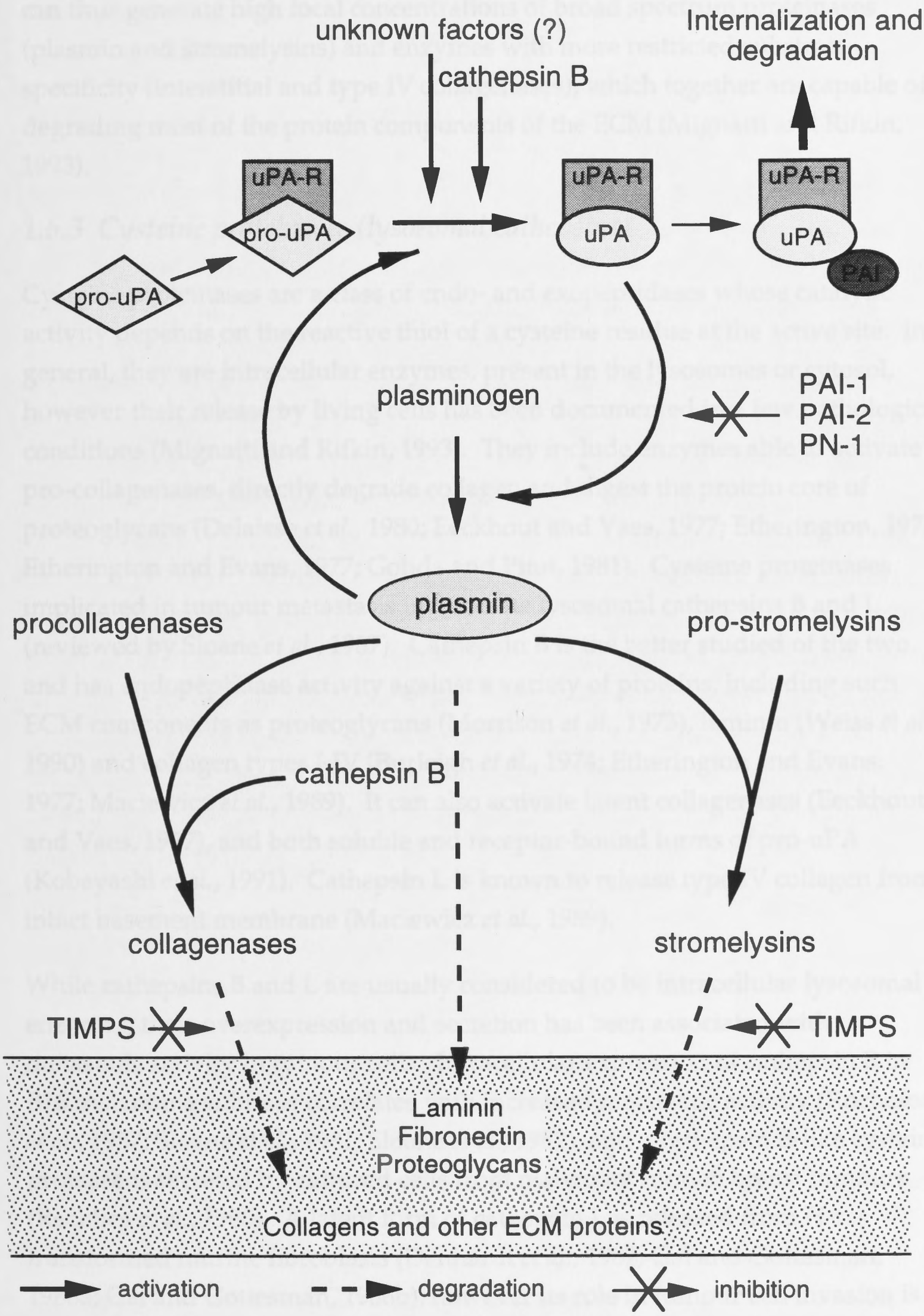


Fig. 1.8 Extracellular control of proteolytic activity

Adapted from Mignatti and Rifkin (1993).

collagenases and pro-stromelysins. Activation of even a small amount of uPA can thus generate high local concentrations of broad spectrum proteinases (plasmin and stromelysins) and enzymes with more restricted substrate specificity (interstitial and type IV collagenases), which together are capable of degrading most of the protein components of the ECM (Mignatti and Rifkin, 1993).

1.6.3 Cysteine proteinases (lysosomal cathepsins)

Cysteine proteinases are a class of endo- and exopeptidases whose catalytic activity depends on the reactive thiol of a cysteine residue at the active site. In general, they are intracellular enzymes, present in the lysosomes or cytosol, however their release by living cells has been documented in a few pathological conditions (Mignatti and Rifkin, 1993). They include enzymes able to activate pro-collagenases, directly degrade collagen and digest the protein core of proteoglycans (Delaisse *et al.*, 1980; Eeckhout and Vaes, 1977; Etherington, 1976; Etherington and Evans, 1977; Gohda and Pitot, 1981). Cysteine proteinases implicated in tumour metastasis include the lysosomal cathepsins B and L (reviewed by Sloane *et al.*, 1987). Cathepsin B is the better studied of the two, and has endopeptidase activity against a variety of proteins, including such ECM components as proteoglycans (Morrison *et al.*, 1973), laminin (Weiss *et al.*, 1990) and collagen types I-IV (Burleigh *et al.*, 1974; Etherington and Evans, 1977; Maciewicz *et al.*, 1989). It can also activate latent collagenases (Eeckhout and Vaes, 1977), and both soluble and receptor-bound forms of pro-uPA (Kobayashi *et al.*, 1991). Cathepsin L is known to release type IV collagen from intact basement membrane (Maciewicz *et al.*, 1989).

While cathepsins B and L are usually considered to be intracellular lysosomal enzymes, their overexpression and secretion has been associated with metastatic activity in tumour cells. Intracellular expression of cathepsin B in B16 melanoma sublines correlates with increased metastatic activity (Rochefort *et al.*, 1987; Sloane *et al.*, 1981; Sloane *et al.*, 1982), and a cathepsin B-like cysteine protease is secreted by cultured cells from malignant human breast tumours (Recklies *et al.*, 1980). A latent form of cathepsin L is released from H-ras transformed murine fibroblasts (Denhardt *et al.*, 1987; Gal and Gottesman, 1986a; Gal and Gottesman, 1986b), however its role in tumour cell invasion is unclear. Both cathepsins are enriched in the plasma membranes of metastatic B16 melanoma cells (Rozhin *et al.*, 1987; Rozhin *et al.*, 1989; Sloane *et al.*, 1986), and cathepsin B in the plasma membranes of malignant and transformed human epithelial cell lines (Krepela *et al.*, 1987; Pietras and Roberts, 1981b;

Sylven *et al.*, 1974), rodent tumour cells (Sloane *et al.*, 1987), squamous carcinoma cells (Pietras and Roberts, 1981a), and an invasive murine bladder carcinoma cell line (Weiss *et al.*, 1990).

It remains unclear whether secreted cathepsins contribute to basement membrane degradation *in vivo*, as they are released as precursors which require proteolytic cleavage to become fully active, and appear in most studies to have limited activity at physiological pH (Briozzo *et al.*, 1988; Denhardt *et al.*, 1987; Maciewicz *et al.*, 1989; Mort and Recklies, 1986; Rochefort, 1990; Rochefort *et al.*, 1990). There are, however, suggestions that tumour cells can modify their surroundings to produce an acidic microenvironment, which may facilitate auto-activation of the pro-enzymes, and one study reported the ability of tumour cells to process secreted pro-enzymes to the mature, active form (Maciewicz *et al.*, 1989). In spite of this, it is questionable whether secreted cathepsin B, whether active or latent, is related to tumour cell invasion, as the levels released do not necessarily correlate with the invasive potential of the cells (Sloane *et al.*, 1987). Several investigators have proposed instead that the plasma membrane-associated form may be more important (Persky *et al.*, 1986; Sloane *et al.*, 1987). Cathepsin B present at the cell surface of tumour cells appears to be in an active form, with a higher pH optimum and greater stability at neutral pH than lysosomal cathepsin B (Rozhin *et al.*, 1987; Weiss *et al.*, 1990), and the specific activity of cathepsins B and L in the plasma membrane fraction of B16 melanoma cells increases with metastatic potential (Rozhin *et al.*, 1989; Sloane *et al.*, 1986).

1.6.4 Glycosidases

Proteoglycan GAG chains are an important structural element of basement membranes. They contribute to the architecture of the basement membrane by enhancing interactions between its protein components (Paulsson *et al.*, 1986) while protecting them against proteolytic attack (Chiarugi, 1982; Nakajima *et al.*, 1988), and to its sieving properties by their polyanionic charge (Yurchenco and Schnittny, 1990). The hydrophilic GAGs form hydrated gels with a huge volume relative to their mass, filling most of the space within the protein meshwork of the ECM (Mignatti and Rifkin, 1993), thus their dissolution in advance of an invading cell should in itself facilitate forward movement, and in addition make the protein scaffold more accessible to the action of proteinases.

Degradation of GAGs requires the action of specific glycosidases, for example, hyaluronidase for hyaluronic acid and heparanase for heparan sulfate (Mignatti

and Rifkin, 1993). Heparan sulfate is the most prominent GAG associated with basement membrane proteoglycans, and so it is significant that a heparanase activity has been associated with metastatic tumour cells. This activity was identified as an endo-β-D-glucuronidase which cleaves heparan sulfate at specific intra-chain sites (reviewed by Nakajima *et al.*, 1988). Release of low M_r heparan sulfate fragments from subendothelial ECM *in vitro* correlates with metastatic potential in B16 melanoma (Kramer *et al.*, 1982; Nakajima *et al.*, 1983; Nakajima *et al.*, 1986; Ricoveri and Cappelletti, 1986), murine lymphoma (Vlodavsky *et al.*, 1983), rat rhabdomyosarcoma (Becker *et al.*, 1986), and murine fibrosarcoma (Ricoveri and Cappelletti, 1986). Furthermore, there is good correlation between the heparanase-inhibitory activity of various heparin preparations and other sulfated polysaccharides, and their anti-metastatic activity *in vivo* (Coombe *et al.*, 1987; Irimura *et al.*, 1986; Parish *et al.*, 1987; Vlodavsky *et al.*, 1988). These data have been taken to implicate heparanase activity in degradation of basement membrane by extravasating tumour cells.

1.6.5 Basement membrane degradation by leukocytes

The invasive behaviour of tumour cells is believed to arise from a loss of control over the invasive phenotype expressed in a restricted manner by normal cells, such as by endothelial cells during angiogenesis, or leukocytes entering inflamed tissues (Stetler-Stevenson *et al.*, 1993). On this basis, emigrating leukocytes are likely to share molecular mechanisms with tumour cells, albeit under different control processes. Mounting evidence suggests that leukocytes, like tumour cells, secrete enzymes capable of degrading basement membrane components.

Several *in vitro* studies have shown that activated murine T cells secrete the serine protease MTSP-1 (murine T cell-specific serine proteinase) (Ebnet *et al.*, 1991; Simon *et al.*, 1991), which degrades type IV collagen (Simon *et al.*, 1991), fibronectin (Simon *et al.*, 1988) and sulfated proteoglycans (Simon *et al.*, 1987; Vettel *et al.*, 1991), and activates pro-uPA (Brunner *et al.*, 1990). A serine protease (HuTSP-1) secreted from activated human T cells (Fruth *et al.*, 1987; Wood *et al.*, 1988) also catalyses the conversion of pro-uPA to its active form, and thus may trigger the enzymatic cascade initiated by plasminogen activation (Brunner *et al.*, 1990). No correlation between these activities and invasive behaviour *in vivo* has actually been demonstrated, however several protease inhibitors reduce the perivascular infiltration of mononuclear cells in a T cell-mediated experimental autoimmune disease (Brosnan *et al.*, 1980).

Activated T cells also secrete a heparanase activity capable of releasing heparan sulfate fragments from ECM *in vitro* (Fridman *et al.*, 1987; Naparstek *et al.*, 1984; Savion *et al.*, 1984; Vettel *et al.*, 1991), as do freshly isolated neutrophils and activated peritoneal macrophages (Matzner *et al.*, 1985; Savion *et al.*, 1987; Savion *et al.*, 1984). *In vivo* studies have implicated heparanase expression in the infiltration of circulating T cells into DTH reactions (Lider *et al.*, 1990), experimental autoimmune lesions and allografts (Lider *et al.*, 1989; Willenborg and Parish, 1988; reviewed by Vlodavsky *et al.*, 1992), highlighting its potential importance in basement membrane degradation.

The degradative enzymes secreted by invasive tumour cells are believed to contribute to a cascade in which multiple proteinases act in concert with one another and/or activate other proteinases with different specificities. Such a spectrum of proteolytic activity has not yet been demonstrated for leukocytes, however MSTP-1 and heparanase appear to act sequentially in the degradation of ECM-associated proteoglycans by T cells *in vitro*. Purified MTSP-1 releases high M_r sulfated proteoglycans from the ECM, which are further degraded into small fragments by the heparanase, while heparanase alone degrades intact ECM poorly (Vettel *et al.*, 1991). Prior proteolysis is also required for release of low M_r heparan sulfate fragments by neutrophil and macrophage heparanases (Matzner *et al.*, 1985; Savion *et al.*, 1987). Thus, the products of proteolysis provide a more accessible substrate for the heparanase. In the context of the intact basement membrane, the two enzymes are proposed to act synergistically, such that proteolytic activity exposes the proteoglycan heparan sulfate side chains, providing a more accessible substrate for the heparanase. In turn, removal of GAG chains from the ECM exposes the collagen network, increasing its susceptibility to proteolysis (David and Bernfield, 1979). Degradation of matrix GAGs could thus facilitate the complete degradation of the ECM by collagenases (Simon *et al.*, 1987; Vettel *et al.*, 1991). In this context, it is significant that purified MTSP-1 degrades purified type IV collagen, and also activates pro-uPA, which contributes to ECM proteolysis by the generation of plasmin from matrix-associated plasminogen. This in turn activates a matrix-associated type IV pro-collagenase (Vettel *et al.*, 1991). Thus, the initial action of MSTP-1 and heparanase on ECM components, combined with their ability to recruit additional proteolytic enzymes, may lead to the complete dissolution of the basement membrane.

1.7 Localization of basement membrane-degrading enzymes

Regulation of ECM degradation is a key concept when considering the invasive behaviour of leukocytes or tumour cells. As described in Section 1.5.3, cell migration through the ECM is thought to proceed by a cyclic process of attachment to ECM components, extension of pseudopodia around which extensive degradation occurs, and movement of the cell into the cleared space. Invasive cells must therefore degrade ECM in a highly organized manner, both spatially and temporally, as uncontrolled lysis of the matrix would remove the substratum needed for continued cell attachment (Liotta, 1986; Liotta, 1990). Several investigators have observed the focal dissolution of fibronectin or reconstituted ECM in regions of contact with adherent cells, both *in vitro* (Chen and Chen, 1987; Chen *et al.*, 1984; Kramer *et al.*, 1986) and *in vivo* (Crissman *et al.*, 1985), with degradation particularly marked adjacent to pseudopodia extended in the direction of cell locomotion. These studies illustrate that degradative activity can be concentrated at discrete points over the cells' surface.

The ability of tumour cells to secrete inhibitors for many of the proteases they produce offers one means for localized control over ECM degradation. Plasminogen activators, for example, are inhibited by PAIs and metalloproteinases by TIMPS. Thus, invading cells may regulate proteolysis by controlling the local balance between enzyme and inhibitor molecules, and varying it at different points on the cell surface. At points of forward protrusion, the balance could be towards net proteolysis, while at points of attachment to the substratum, proteolysis might be inhibited (Liotta, 1990). In support of this, Chen (1992) observed basement membrane degradation by transformed cells at sites of contact with pseudopodia but not at adhesive foci, and found PAI-1 at sites of attachment but not on pseudopodia. Thus, PAIs may be differentially expressed across the cell surface, so as to minimize ECM degradation at sites of adhesion, and to permit it where degradation is required.

A second means of controlling the extent of ECM degradation is offered by the cell surface expression of hydrolytic enzymes, thereby limiting cleavage of matrix components to the immediate vicinity of the cell. Enzyme activity could be localised further by restricting expression to specific parts of the cell membrane, much as was suggested for enzyme inhibitors. While some studies have demonstrated degradative activity in the conditioned medium of

activated leukocytes (Fridman *et al.*, 1987; Vettel *et al.*, 1991; Vlodavsky *et al.*, 1992), others have shown matrix degradation to require contact between cells and ECM. Parish *et al.* (1992) found that while intact or heat-killed metastatic rat mammary adenocarcinoma cells degraded ECM *in vitro*, sonicated or detergent-solubilized cells, and culture supernatants did not. Likewise, ECM degradation by B16 melanoma cells (Kramer *et al.*, 1982) and transformed chicken fibroblasts (Chen *et al.*, 1984) was not reproduced by their conditioned medium, suggesting that degradation was due to membrane-associated enzymes rather than secretory products.

Cell surface expression of proteinases by metastatic tumour cells has been described in many studies. In the case of serine proteinases, pro-uPA is activated while bound to a cell surface receptor and remains associated with it for a prolonged period (Section 1.6.1). Plasmin is thus generated in close proximity to the cell surface, and in turn activates several latent MMPs (Stetler-Stevenson *et al.*, 1993). The 72 kDa gelatinase is also activated at the cell surface, in the context of a poorly defined receptor/activator system (Kleiner and Stetler-Stevenson, 1993). MMP activities able to degrade collagens, gelatin and fibronectin are highly enriched in plasma membrane fractions isolated from metastatic tumour cell lines of rat, murine and human origin (Chen and Chen, 1987; Chen *et al.*, 1984; DiStefano *et al.*, 1982; DiStefano *et al.*, 1983; Zucker and Lysik, 1977; Zucker *et al.*, 1985a; Zucker *et al.*, 1985b; Zucker *et al.*, 1987), and appear to be integral membrane proteins (Zucker *et al.*, 1987). Active forms of cathepsins B and L are also associated with the cell surface (Boyer and Tannock, 1993; Sloane *et al.*, 1987).

1.8 A role for the phosphomannosyl recognition system in cell surface expression of lysosomal hydrolases

While it is recognised that cell surface expression of several classes of proteinase may be an important aspect of invasive behaviour, mechanisms for this have not been defined in many cases. In fact, in the case of cathepsin B, there are conflicting reports regarding its cell surface expression on invasive cells. Cysteine proteinase activity in human lung cancer cells appears to be only loosely associated with the plasma membrane (Zucker *et al.*, 1987), however attempts to isolate cathepsin B from the plasma membrane fraction of B16 melanoma cells suggested it to be an integral membrane protein (Rozhin *et al.*, 1987). Cytochemical staining of human breast carcinoma cells (Krepela *et al.*, 1987) and B16 melanoma cells (Sloane *et al.*, 1990) subsequently showed it to be associated with vesicles located at the periphery of the cells, and extending

out along cytoplasmic projections, and a later study located it on the outer surface of B16 melanoma cells (Sloane *et al.*, 1991). These investigators suggested that cathepsins B and L might both be secreted from peripheral vesicles in response to an acidic microenvironment, but did not speculate further on means of attachment to the cell surface.

A mechanism for the cell surface expression of lysosomal hydrolases has been described in detail. Central to this mechanism are the mannose 6-phosphate receptors (MPRs), which mediate intracellular transport of lysosomal enzymes to the lysosomes (reviewed by Kornfeld, 1992). These receptors recognise a mannose 6-phosphate (M6P) marker common to all lysosomal enzymes, and transport newly synthesized enzymes from the Golgi apparatus to an acidified prelysosomal compartment, from which they are packaged into the lysosomes. MPRs are also expressed at the cell surface, where they function in the binding and endocytosis of exogenous lysosomal enzymes. Parish *et al.* (1990) have proposed that lysosomal enzymes secreted from the cell and bound by cell surface MPRs may contribute to the cell surface-associated degradative activity of cells with invasive potential.

This mechanism probably does not apply to the cell surface expression of the lysosomal cathepsins on metastatic tumour cells, as cathepsin B associated with the plasma membrane fraction of B16 melanoma cells was not displaced by M6P, a competitive inhibitor of the MPR-lysosomal enzyme interaction (Rozhin *et al.*, 1987), and cathepsin L secreted from transformed cells binds to MPR with low affinity, due to a defective M6P marker (Dong and Sahagian, 1990). However, while the cysteine proteases are the only class of lysosomal enzyme so far implicated in basement membrane degradation, the lysosomes are a rich source of enzymes with proteolytic and glycolytic activity, and many digest internalized ECM fragments in the context of the lysosomes (Kaiser, 1980). There may be other lysosomal enzymes that, like the cathepsins, are capable of acting in the extracellular environment, and may participate in the degradation of intact ECM.

(Brauker et.al. 1986)

In general support of this hypothesis, Wang and coworkers have demonstrated that lysosomal hydrolases bound to cell surface MPRs degrade cell surface and substratum-attached proteoglycans. Addition of M6P to human fibroblast cultures inhibited degradation of extracellular ^{35}S-labelled proteoglycans, but had no effect on proteoglycan turnover by fibroblasts from patients with I-cell disease, in which lysosomal enzymes lack the M6P marker. Furthermore, addition of "normal" lysosomal enzymes to I cells enhanced proteoglycan

degradation, an increase that was inhibitable with M6P. If lysosomal enzymes were active in solution, addition of M6P, which releases the enzymes from cell surface receptors, should have enhanced proteoglycan degradation by fibroblasts. Instead, these studies suggested that cell surface MPR anchored these enzymes in close proximity to pericellular and extracellular substrates (Brauker *et al.*, 1986; Kornfeld, 1992; Roff *et al.*, 1982).

Parish and coworkers initially tested their hypothesis using the animal disease model Experimental Autoimmune Encephalomyelitis (EAE). EAE is an inflammatory autoimmune disease of the central nervous system (CNS), produced in laboratory animals by immunization with neural tissue in adjuvant (Hashim *et al.*, 1980; Raine, 1984). The host animal mounts a cell-mediated attack on constituents of CNS myelin, the target antigen being a peptide found in the basic protein (BP) fraction (Hashim *et al.*, 1978). The disease is characterised by leukocyte infiltration of the brain and spinal cord, oedema and fibrin deposition (Raine, 1984; Simmons *et al.*, 1987). The influx of leukocytes, predominantly lymphocytes and macrophages, results in characteristic perivenular lesions. Inflammation in the CNS, predominantly in the lower spinal cord, leads to paresis and paralysis of the hind limbs. EAE can be induced passively by intravenous transfer of activated T cells, either from actively immunized donors, or MBP-specific T cell lines, to naive syngeneic recipients (Astrom and Waksman, 1962; Ben-Nun *et al.*, 1981; Panitch and McFarlin, 1977; Paterson, 1960). Adoptively transferred EAE has been utilized in *in vivo* studies of lymphocyte extravasation, as development of disease is dependent on the passage of transferred T cells from the circulation into the CNS.

Parish and coworkers demonstrated that several compounds which interfere with the lysosomal enzyme-MPR interaction inhibited the development of EAE in rats. M6P and its structural analogue fructose-1-phosphate (F1P), which competitively inhibit the binding of lysosomal enzymes to MPRs (Kaplan *et al.*, 1977a), reduced clinical signs of disease by over 80%. The number of spinal cord lesions was reduced dramatically, and in some animals completely (Willenborg *et al.*, 1989b). In contrast, D-mannose, which has low affinity for MPR (Kaplan *et al.*, 1977a), failed to inhibit disease. The plant alkaloid castanospermine (CS), an inhibitor of glycoprotein processing which prevents the formation of the M6P recognition marker on lysosomal enzymes, also inhibited clinical signs of EAE (Willenborg *et al.*, 1989a). CS did not reduce the number of inflammatory lesions in the CNS, however these were intense and

compact, with leukocytes tightly packed around the margins of vessels and little migration into the parenchyma compared to controls. The inhibitory effects of M6P and CS were not limited to T cell entry into the CNS, as these compounds also reduced joint inflammation in passively induced adjuvant arthritis (Willenborg *et al.*, 1992).

These experiments support the hypothesis that MPRs provide a means of displaying lysosomal enzymes at the cell surface, and that these contribute to the degradation of the subendothelial basement membrane by extravasating leukocytes. The absence of lesions in the M6P-treated animals suggests that adoptively-transferred T lymphocytes were completely prevented from leaving the circulation. In the case of CS, cells were able to cross the vascular endothelium, indicating that the adhesion and transmigration processes were not affected, but then appeared to accumulate before some limiting structure, possibly the basement membrane (Willenborg *et al.*, 1989a). The effect of CS on CNS lesions highlights the concept that lysosomal enzymes may be most effective when displayed on the cell surface via MPRs. Since CS prevents formation of the M6P marker, newly synthesized lysosomal enzymes are unable to bind to MPRs in the Golgi, resulting in secretion of these enzymes via the secretory pathway (Hickman and Neufeld, 1972). If soluble lysosomal enzymes were effective in degrading basement membrane, CS should have enhanced leukocyte entry into the CNS. However, although the infiltrating lymphocytes in the EAE and arthritis models may have secreted lysosomal enzymes in unusually large amounts, their entry into the CNS parenchyma was reduced.

These experiments support the proposal that MPR-mediated expression of lysosomal enzymes at the cell surface is an important feature of leukocyte migration. The aim of this thesis was to further examine the hypothesis that lysosomal enzymes in association with cell surface MPRs are involved in basement membrane degradation. The remainder of this review will focus on MPRs and the phosphomannosyl recognition system, as this forms the basis of the experimental work described in this thesis.

1.9 The phosphomannosyl recognition system: an overview

Most lysosomal enzymes are soluble glycoproteins that are synthesized on the membrane-bound ribosomes of the rough endoplasmic reticulum (ER), together with secretory proteins, membrane proteins and proteins destined for the lumens of the ER and Golgi apparatus. The various classes of protein are

transported from the ER lumen to the Golgi, from which each is directed to it's correct destination. As soluble proteins are mixed together in the lumen of the Golgi, this requires their physical separation from one another. Segregation of lysosomal enzymes from other soluble proteins is effected via the phosphomannosyl recognition system. During their passage through the ER and Golgi apparatus, lysosomal enzymes selectively acquire asparagine-linked, high mannose-type oligosaccharides with terminal mannose 6-phosphate (M6P) residues. These bind with high affinity to M6P receptors (MPRs) in the Golgi membranes, effectively separating lysosomal enzymes from other soluble proteins which remain in the lumen. MPR-enzyme complexes are then gathered into clathrin-coated pits which bud off from the Golgi, forming coated vesicles which fuse with an acidified endosomal/prelysosomal compartment. The receptor-enzyme complex dissociates at low pH, and the freed enzymes are packaged into lysosomes, where acid phosphatase inactivates the M6P marker by removing the phosphate groups. The MPRs recycle back to the Golgi to repeat the process, or to the plasma membrane where they function in endocytosis of extracellular lysosomal enzymes.

A significant percentage of most lysosomal enzymes escapes direct lysosomal delivery and is secreted; these enzymes may be recaptured by cell surface MPR. MPR-ligand complexes on the plasma membrane are also concentrated into coated pits where they are internalised and delivered to the same acidic prelysosomal compartment, which forms the intersection point between the biosynthetic and endocytic transport pathways (Fig. 1.9). The phosphomannosyl recognition system has been reviewed by Dahms *et al.* (1989), Kornfeld (1986), Kornfeld (1987), Kornfeld (1992), Kornfeld and Mellman (1989), Pfeffer (1991), Sly and Fischer (1982), and von Figura and Hasilik (1986).

Three key processes contribute to lysosomal enzyme targeting via the phosphomannosyl recognition system:

a) selective modification of lysosomal enzymes by Golgi enzymes which synthesize the M6P recognition marker
b) recognition of this marker by MPRs
c) recognition of MPRs by cellular components that mediate the selective transport of MPR-enzyme complexes to lysosomes (Kornfeld and Mellman, 1989).

Each of these processes will be considered in this review.

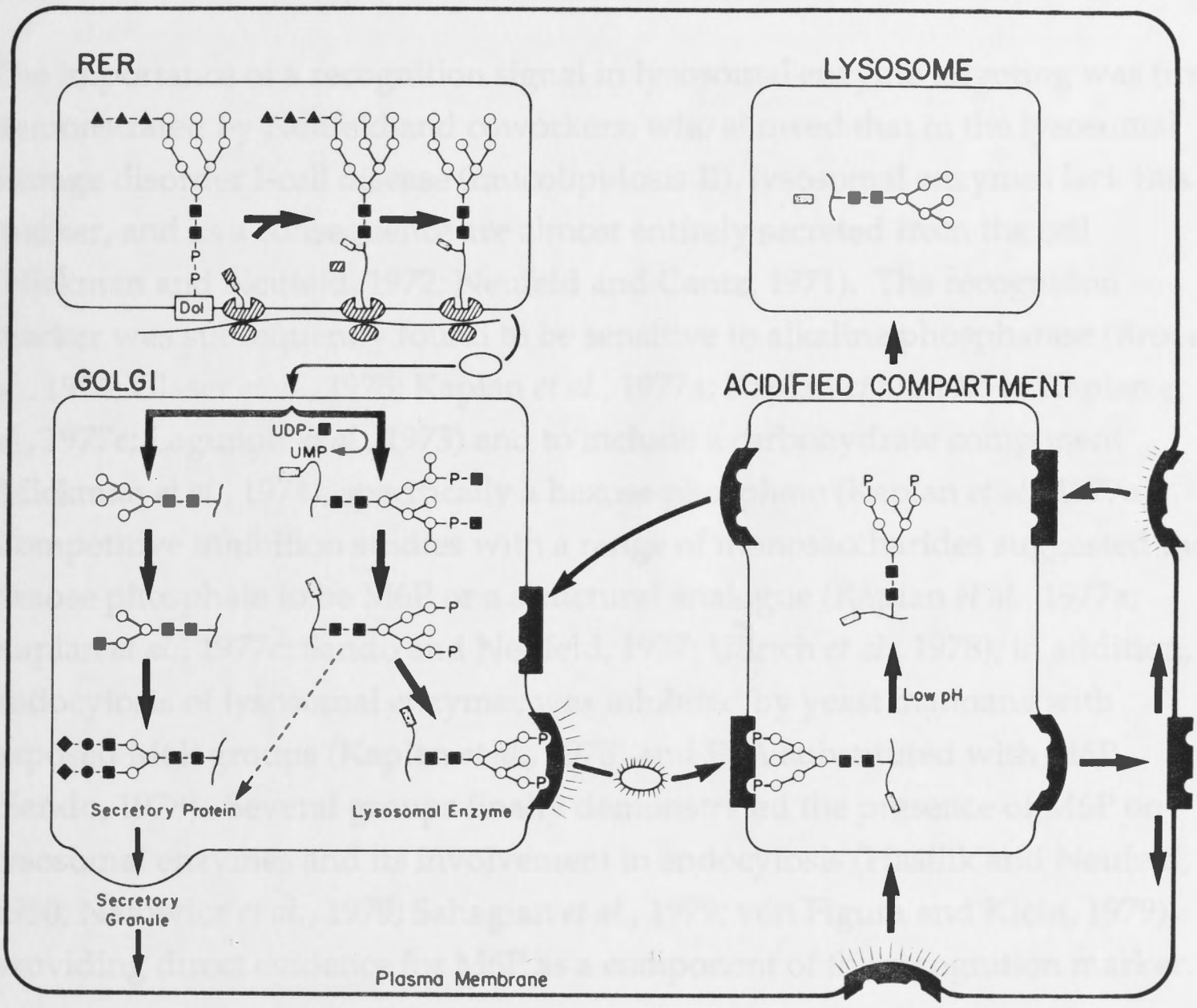


Fig. 1.9 Schematic pathway of lysosomal enzyme targeting to lysosomes.

Lysosomal enzymes and secretory proteins are synthesized in the rough endoplasmic reticulum (RER) and glycosylated by the transfer of a preformed oligosaccharide from dolichol-P-P-oligosaccharide (Dol). The newly synthesized proteins are translocated to the Golgi where the oligosaccharides of secretory proteins are processed to complex-type units and the oligosaccharides of lysosomal enzymes are phosphorylated on terminal mannose residues. Most of the lysosomal enzymes bind to mannose 6-phosphate receptors (MPRs) (▬▬) and are tranported to an acidified prelysosomal compartment where the receptor-ligand complex dissociates. The MPRs recycle back to the Golgi or to the cell surface, while the enzymes are packaged into lysosomes. A small number of lysosomal enzymes fail to bind to the receptors in the Golgi, and are secreted via the secretory pathway (- - ►). These enzymes bind to cell surface MPR in coated pits (◡◡◡), and are internalized to the prelysosomal compartment.

(■) N-acetylglucosamine; (○) mannose; (▲) glucose; (●) galactose; (◆) sialic acid.

From Kornfeld (1987).

1.10 The M6P recognition marker

The importance of a recognition signal in lysosomal enzyme targeting was first demonstrated by Neufeld and coworkers, who showed that in the lysosomal storage disorder I-cell disease (mucolipidosis II), lysosomal enzymes lack this marker, and as a consequence are almost entirely secreted from the cell (Hickman and Neufeld, 1972; Neufeld and Cantz, 1971). The recognition marker was subsequently found to be sensitive to alkaline phosphatase (Brot *et al.*, 1974; Glaser *et al.*, 1975; Kaplan *et al.*, 1977a; Kaplan *et al.*, 1977b; Kaplan *et al.*, 1977c; Lagunoff *et al.*, 1973) and to include a carbohydrate component (Hickman *et al.*, 1974), specifically a hexose phosphate (Kaplan *et al.*, 1977a). Competitive inhibition studies with a range of monosaccharides suggested the hexose phosphate to be M6P or a structural analogue (Kaplan *et al.*, 1977a; Kaplan *et al.*, 1977c; Sando and Neufeld, 1977; Ullrich *et al.*, 1978); in addition, endocytosis of lysosomal enzymes was inhibited by yeast mannans with exposed M6P groups (Kaplan *et al.*, 1978) and BSA substituted with M6P (Sando, 1978). Several groups finally demonstrated the presence of M6P on lysosomal enzymes and its involvement in endocytosis (Hasilik and Neufeld, 1980; Natowicz *et al.*, 1979; Sahagian *et al.*, 1979; von Figura and Klein, 1979), providing direct evidence for M6P as a component of the recognition marker.

1.10.1 Biosynthesis of the M6P marker

The structure and biosynthesis of the lysosomal enzyme recognition marker are now well characterized (reviewed by Kornfeld (1986), Kornfeld (1987), Kornfeld (1990), Kornfeld and Mellman (1989), Pfeffer (1991), and Sly and Fischer (1982)). Asparagine-linked oligosaccharides added to lysosomal enzymes in the ER are selectively modified in the Golgi apparatus, producing a high-mannose structure with one or two terminal M6P residues (Fig. 1.10). The early oligosaccharide processing steps are shared with secretory and membrane proteins, while the later ones are unique to lysosomal enzymes.

Proteins within the ER are glycosylated by the co-translational transfer of a preformed oligosaccharide from a dolichol pyrophosphate carrier to the asparagine residue of an Asn-X-Ser/Thr sequence. The oligosaccharide consists of three glucose, nine mannose (man) and two N-acetylglucosamine (GNAc) residues. This undergoes a series of trimming and modification reactions, with the end result dependent on the protein moiety. The initial trimming reactions take place in the ER. The first glucose residue is rapidly removed by glucosidase I, and the remaining ones more slowly by glucosidase

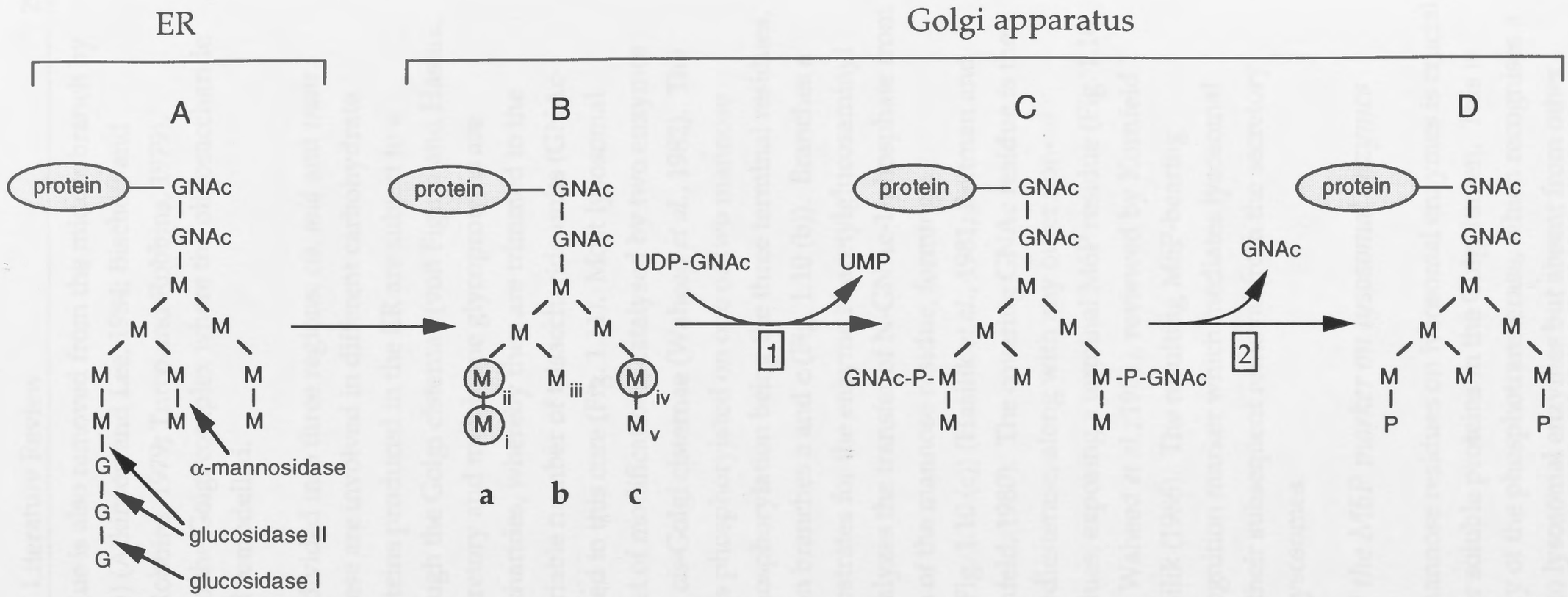


Fig. 1.10 Synthesis of the M6P marker.

The main stages in processing of high mannose oligosaccharides on lysosomal enzymes. Three glucose and one mannose residue are trimmed from N-linked oligosaccharides in the endoplasmic reticulum (ER) (A). In the Golgi, the oligosaccharide is phosphorylated on either one or two mannose residues. Potential phosphorylation sites are marked i-v in (B). Of these five sites, three are preferred (◯). Residues i and ii are phosphorylated most frequently, although never simultaneously, followed by residue iv. Residue v is the least common phosphorylation site. Phosphorylation occurs in a two step process: P-GNAc is transferred from UDP-GNAc to the mannose residue (C), and GNAc is removed (D). The enzymes involved are (1) UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-phosphotransferase and (2) α-N-acetylglucosaminyl phosphodiester glycosidase. Outer non-phosphorylated mannose residues are also removed, exposing terminal M6P residues (Varki and Kornfeld, 1980; Varki and Kornfeld, 1983).

Abbreviations: G (glucose), M (mannose), GNAc (N-acetylglucosamine), P (phosphate).

II. The terminal mannose residue is also removed from the middle branch by an α-mannosidase (Fig. 1.10 (a)) (Atkinson and Lee, 1984; Bischoff and Kornfeld, 1983; Hubbard and Robbins, 1979; Turco and Robbins, 1979). Proteins are then transported to the Golgi complex where the oligosaccharide chains are further trimmed and remodelled.

Functionally, the Golgi can be divided into three regions: *cis*, *mid* and *trans*. While not rigidly separated, these are involved in different carbohydrate modification reactions and proteins produced in the ER are subject to a vectorial, *cis* to *trans*, flow through the Golgi cisternae (von Figura and Hasilik, 1986). Oligosaccharides on secretory and membrane glycoproteins are processed to complex oligosaccharides, whereby they are trimmed to the innermost 5 residues, and a variable number of trisaccharide units (GNAc-galactose-sialic acid) are attached to this core (Fig. 1.11). Most lysosomal enzymes undergo a different set of modifications, catalysed by two enzymes found in the membranes of the *cis*-Golgi cisternae (Waheed *et al.*, 1982). The $man_8\ GNAc_2$ oligosaccharide is phosphorylated on one or two mannose residues, those available for phosphorylation being the three terminal residues, and the penultimate residues on branches a and c (Fig. 1.10 (b)). Branches a and c are the most frequent substrates for the enzyme N-acetylglucosaminyl phosphotransferase, which catalyzes the transfer of N-GNAc-1-phosphate from UDP-GNAc to the C-6 position of the mannose residue, forming a phosphodiester intermediate (Fig. 1.10 (c)) (Hasilik *et al.*, 1981; Reitman and Kornfeld, 1981; Varki and Kornfeld, 1980). The covering GNAc residue is then removed by a specific phosphodiesterase, along with any outer non-phosphorylated mannose residues, exposing a terminal M6P residue (Fig. 1.10 (d)) (Varki and Kornfeld, 1981; Waheed *et al.*, 1981), reviewed by Kornfeld (1990) and von Figura and Hasilik (1986). The resulting M6P-bearing oligosaccharide acts as the recognition marker which mediates lysosomal enzyme binding to MPRs and their subsequent removal from the secretory pathway and translocation to lysosomes.

1.10.2 Selective formation of the M6P marker on lysosomal enzymes

Selective phosphorylation of mannose residues on lysosomal enzymes is crucial for their segregation from other soluble proteins in the Golgi lumen. This is achieved through the specificity of the phosphotransferase, which recognises a protein determinant common to lysosomal enzymes but absent from other glycoproteins (Lang *et al.*, 1984; Reitman and Kornfeld, 1981; Waheed *et al.*, 1982). By using fragments of lysosomal cathepsin D to generate a

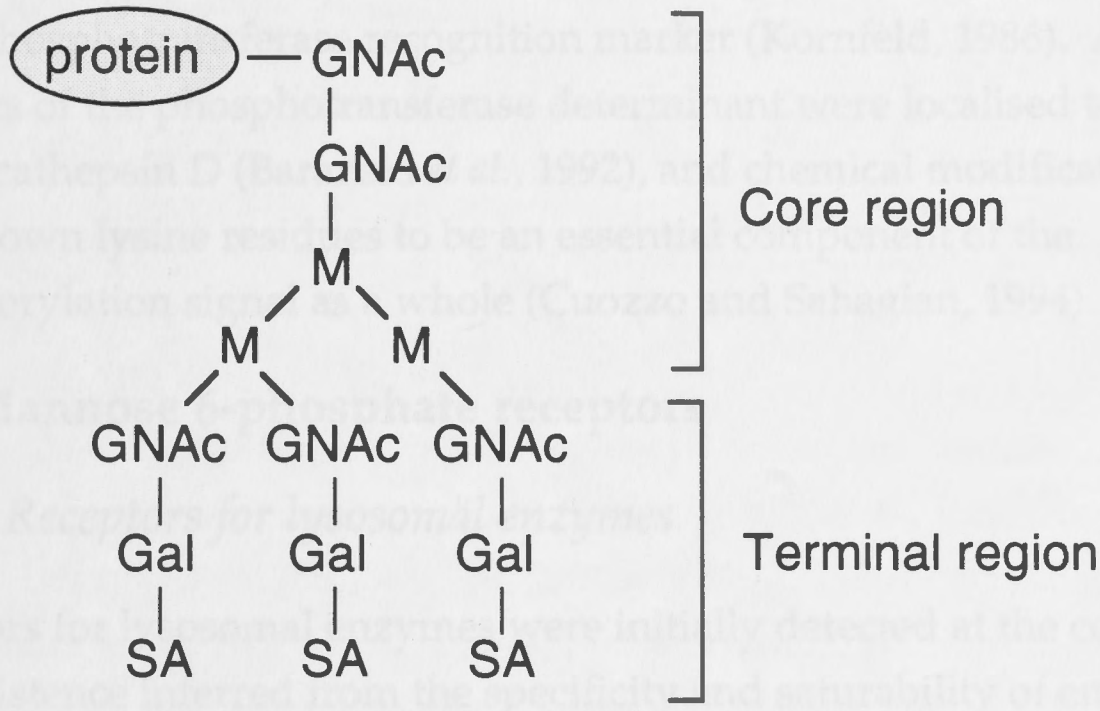


Fig. 1.11 General structure of complex oligosaccharides

High mannose N-linked oligosaccharides are trimmed by ER and Golgi enzymes, leaving a core region of two N-GNAc and three mannose residues. The terminal region consists of GNAc-galactose-sialic acid trisaccharides linked to a variable number of the core mannose residues. GNAc, galactose and sialic acid are added sequentially, in different regions of the Golgi. The terminal region may be truncated, consisting of disaccharides (GNAc-galactose), or GNAc only (Alberts *et al.*, 1986).

Abbreviations: GNAc (N-acetylglucosamine), M (mannose), Gal (galactose), SA (sialic acid).

phosphotransferase recognition site in the related secretory protein pepsinogen, Baranski *et al.* (1990) showed that two non-continuous sequences from the carboxyl lobe of cathepsin D (residues 188-230, particularly lysine 203, and 265-292) are critical elements of this determinant. X-ray crystallography showed that the two sequences are located alongside one another on the surface of the protein, consistent with earlier studies showing the conformational dependence of the phosphotransferase recognition marker (Kornfeld, 1986). Additional elements of the phosphotransferase determinant were localised to the amino lobe of cathepsin D (Baranski *et al.*, 1992), and chemical modification studies have shown lysine residues to be an essential component of the phosphorylation signal as a whole (Cuozzo and Sahagian, 1994).

1.11 Mannose 6-phosphate receptors

1.11.1 Receptors for lysosomal enzymes

Receptors for lysosomal enzymes were initially detected at the cell surface, their existence inferred from the specificity and saturability of enzyme uptake by fibroblasts (Fischer *et al.*, 1980a), and the demonstration of cell surface binding sites for α-L-iduronidase (Rome *et al.*, 1979) and β-glucuronidase (Gonzalez-Noriega *et al.*, 1980). Evidence for intracellular receptors was provided by Fischer *et al.* (1980a), who found receptors for lysosomal enzymes on the internal membranes of fibroblasts. A lysosomal enzyme receptor was finally isolated from bovine liver on the basis of its affinity for β-galactosidase (Sahagian *et al.*, 1981), and was found to be an integral membrane glycoprotein with apparent M_r of 215 000 by SDS-PAGE, with high affinity for both lysosomal enzymes and M6P (Fischer *et al.*, 1982; Sahagian *et al.*, 1981; Steiner and Rome, 1982).

While the 215, 000-M_r receptor was found to be widely distributed, it was absent from a number of rodent cell lines, such as P388D_1 and J774 macrophages, L cells (fibroblasts), MOPC315 (myeloma cells) (Gabel *et al.*, 1983) and Morris hepatoma 7777 (Mainferme *et al.*, 1985). These cells were still able to direct lysosomal enzymes to lysosomes, inferring the existence of an alternative pathway for lysosomal enzyme targeting (Gabel *et al.*, 1983). Hoflack and Kornfeld (1985a) identified a second lysosomal enzyme receptor on P388D_1 membranes, which required the presence of divalent cations for ligand binding *in vitro*. This receptor has since been purified from a variety of tissues (Distler and Jourdian, 1987; Hoflack and Kornfeld, 1985b; Stein *et al.*, 1987a; Stein *et al.*, 1987c),and shown to be an integral membrane glycoprotein

with an apparent subunit M_r of approximately 46, 000. On the basis of their differential requirements for divalent cations, the larger receptor has frequently been referred to as the cation-independent M6P receptor (CI-MPR,) and the smaller receptor as the cation-dependent M6P receptor (CD-MPR). In this review, they will be referred to as MPR-300 and MPR-46 respectively, on the basis of their calculated M_r.

1.11.2 The cation-independent MPR (MPR-300)

1.11.2.1 Sequence and structure

MPR-300 is a large, type I transmembrane glycoprotein (Sahagian and Steer, 1985; von Figura *et al.*, 1985), and a schematic diagram of its structure is shown in Fig. 1.12. Complete amino acid sequences have been deduced from cDNA clones for the human (Morgan *et al.*, 1987; Oshima *et al.*, 1988), bovine (Lobel *et al.*, 1987; Lobel *et al.*, 1988), murine (Ludwig *et al.*, 1994) and rat receptors (MacDonald *et al.*, 1988), and show 80-90% homology between species. The protein component has a calculated mass of 275 kDa, and is 2491/2499 amino acid residues long (human /bovine). The newly synthesized receptor is guided into the lumen of the rough ER by an N-terminal signal sequence of 40/44 residues. A single hydrophobic stretch of 23 amino acid residues divides the receptor into cytoplasmic and extracytoplasmic domains of 164/163 and 2265/2269 residues, respectively (Lobel *et al.*, 1988; Morgan *et al.*, 1987; Oshima *et al.*, 1988; von Figura *et al.*, 1985). The M6P binding site is located on the N-terminal extracytoplasmic domain (Sahagian and Steer, 1985), which is extensively cross-linked by disulfide bonds (Sahagian and Neufeld, 1983), and contains 19 potential asparagine-linked glycosylation sites, at least two of which are glycosylated with complex-type oligosaccharides (Lobel *et al.*, 1987; Sahagian and Neufeld, 1983). The smaller cytoplasmic domain contains only two potential glycosylation sites (Lobel *et al.*, 1988). Glycosylation yields a mature receptor of up to 300 kDa (Lobel *et al.*, 1988).

The extracytoplasmic domain has a highly repetitive structure, consisting of 15 homologous repeat sequences of 134-167 amino acid residues, followed by a 23-residue "stalk" adjacent to the transmembrane region (Lobel *et al.*, 1987; Lobel *et al.*, 1988). The repeating units share an overall 16-38% sequence identity, as well as regions of conservatively substituted amino acids (Dahms *et al.*, 1989). Most notable is a distinctive pattern of 8 conserved cysteine residues, all of which participate in intrachain disulfide bonds. Each repeat also contains a highly conserved 13-amino acid stretch that matches the consensus sequence

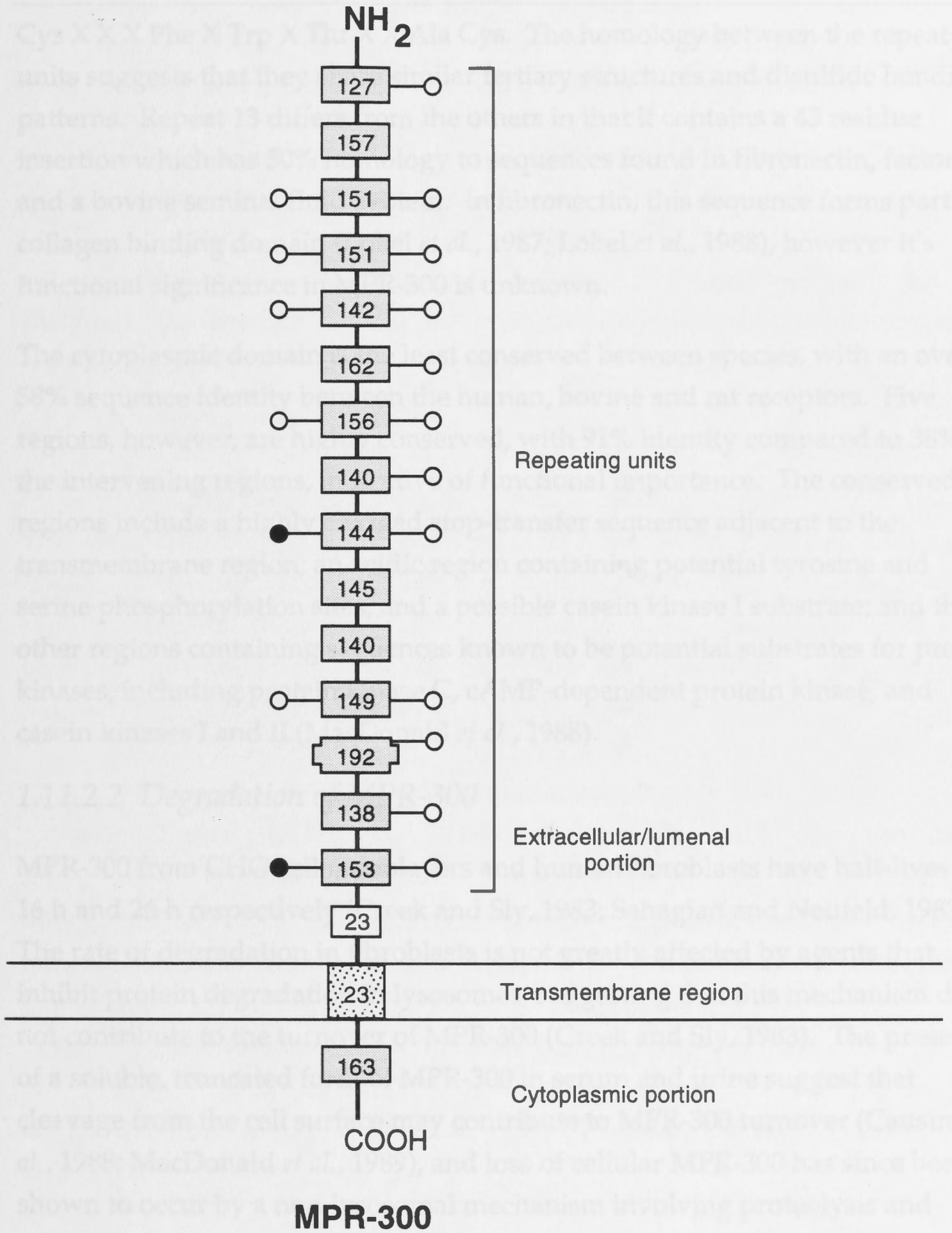


Fig. 1.12 Schematic representation of MPR-300.

Segmentation of the extracellular portion of MPR-300 into domains is based on optimal alignment of repeating units. The number of amino acid residues in each domain is shown. The insertion in domain 13 represents the region that is similar to sequences found in fibronectin and factor XII. Closed circles represent N-linked glycosylation sites known to be used, while open circles represent potential glycosylation sites

Modified from Kornfeld (1987).

Cys X X X Phe X Trp X Thr X X Ala Cys. The homology between the repeat units suggests that they share similar tertiary structures and disulfide bonding patterns. Repeat 13 differs from the others in that it contains a 43 residue insertion which has 50% homology to sequences found in fibronectin, factor XII and a bovine seminal fluid protein. In fibronectin, this sequence forms part of a collagen binding domain (Lobel *et al.*, 1987; Lobel *et al.*, 1988), however it's functional significance in MPR-300 is unknown.

The cytoplasmic domain is the least conserved between species, with an overall 58% sequence identity between the human, bovine and rat receptors. Five regions, however, are highly conserved, with 91% identity compared to 38% for the intervening regions, indicative of functional importance. The conserved regions include a highly charged stop-transfer sequence adjacent to the transmembrane region; an acidic region containing potential tyrosine and serine phosphorylation sites, and a possible casein kinase I substrate; and three other regions containing sequences known to be potential substrates for protein kinases, including protein kinase C, cAMP-dependent protein kinase, and casein kinases I and II (MacDonald *et al.*, 1988).

1.11.2.2 Degradation of MPR-300

MPR-300 from CHO cell monolayers and human fibroblasts have half-lives of 16 h and 26 h respectively (Creek and Sly, 1983; Sahagian and Neufeld, 1983). The rate of degradation in fibroblasts is not greatly affected by agents that inhibit protein degradation in lysosomes, suggesting that this mechanism does not contribute to the turnover of MPR-300 (Creek and Sly, 1983). The presence of a soluble, truncated form of MPR-300 in serum and urine suggest that cleavage from the cell surface may contribute to MPR-300 turnover (Causin *et al.*, 1988; MacDonald *et al.*, 1989), and loss of cellular MPR-300 has since been shown to occur by a non-lysosomal mechanism involving proteolysis and release into the extracellular fluid (Clairmont and Czech, 1991).

1.11.2.3 Binding to M6P-bearing ligands

MPR-300 has two high affinity M6P binding sites per molecule (Tong *et al.*, 1989). As each of the 15 repeat units of MPR-300 is homologous to the extracytoplasmic domain of MPR-46 (Dahms *et al.*, 1987), which contains a single M6P binding site (Section 1.11.3.3), the two M6P binding sites on MPR-300 were each proposed to occupy a single repeat unit (Tong *et al.*, 1989). They have since been localized to repeats 1-3 and 7-9 (Westlund *et al.*, 1991), and site-

directed mutagenesis has shown that arginine 435 (repeat 3) and arginine 1334 (repeat 9) are essential components of the binding sites (Dahms *et al.*, 1993).

MPR-300 has maximum affinity for M6P-bearing ligands at pH 6.0-6.3. Binding decreases slowly with increasing pH, but very rapidly below pH 6.0, such that M6P binding at pH 5.5 is less than 25% of that at pH 6.0 (Tong *et al.*, 1989). This pH range is well suited to allow ligand binding in the Golgi and at the cell surface, while favouring ligand release in the acidic prelysosomal/endosomal compartment.

1.11.2.4 Ligand characterization

MPR-300 has high affinity for oligosaccharides bearing a terminal M6P residue, and lower affinity for phosphodiesters, such as found on lysosomal enzymes from the slime mold *Dictyostelium discoideum* (Kaplan *et al.*, 1978; Tong *et al.*, 1989). While M6P makes the major contribution, other structural features of the high mannose oligosaccharide also contribute to the binding interaction, as oligosaccharides bearing a single phosphomonoester have higher affinity for MPR-300 than do either M6P or pentamannose phosphate (PMP). The major structural difference is that in the high mannose oligosaccharide, M6P is most commonly linked α1,2 to the underlying mannose residue, whereas the linkage is α1,3 in PMP, and there is no underlying mannose in M6P (Tong *et al.*, 1989; Varki and Kornfeld, 1980). In this context, the disaccharide man-α1,2-man inhibits lysosomal enzyme binding 17 times more potently than does mannose (Distler *et al.*, 1979). Thus, MPR-300 may recognise an extended oligosaccharide structure which includes the M6P-α1,2-mannose sequence, or alternatively, M6P linked α1,2 to the underlying mannose may adopt a more favourable conformation for binding than M6P alone or with an α1,3 linkage (Tong *et al.*, 1989).

The presence of two M6P binding sites on MPR-300 suggests that divalent interactions may occur between a single receptor molecule and ligands bearing more than one phosphomannosyl group. In support of this, lysosomal enzymes, and oligosaccharides carrying two terminal M6P residues, have greater affinity for MPR-300 than oligosaccharides with only one M6P (Tong *et al.*, 1989; Varki and Kornfeld, 1983). The binding affinity of divalent ligands is lower than that predicted for binding to two non-interacting sites, however, possibly because the relative positioning of the two M6P residues on the oligosaccharide do not precisely match the positioning of the two binding sites, resulting in a sub-optimal interaction (Tong *et al.*, 1989). This point raises the

question of whether or not M6P residues mediating divalent interactions between lysosomal enzymes and MPR-300 are located on the same oligosaccharide (Kornfeld, 1992). Of interest in this regard is cathepsin L, a lysosomal enzyme which is secreted in large amounts by virally-transformed mouse fibroblasts (Dong *et al.*, 1989), seemingly due to an unusually low affinity for MPR-300. Significantly, it carries a single oligosaccharide with two terminal M6P residues, while cathepsin L produced by CHO cells bears two oligosaccharides, each with two M6P residues, and binds MPR-300 with high affinity (Dong and Sahagian, 1990). This suggests that M6P residues located on separate oligosaccharides may interact more favourably with MPR-300 than do two M6P residues on the same oligosaccharide (Kornfeld, 1992).

1.11.2.5 Identity with the insulin-like growth factor II receptor

The relationship between MPR-300 and the insulin-like growth factor II (IGF-II) receptor became apparent when Morgan *et al.* (1987) sequenced the human IGF-II receptor from full length cDNA clones and found it 80% identical to a partial clone of bovine MPR-300 (Lobel *et al.*, 1987). It was subsequently shown to be 99% identical to human MPR-300 (Oshima *et al.*, 1988). Expression of these essentially identical cDNAs resulted in the appearance of both cell surface IGF-II receptors (Morgan *et al.*, 1987) and a receptor able to mediate β-glucuronidase uptake (Oshima *et al.*, 1988). Furthermore, the purified IGF-II receptor was recognised by two different polyclonal antibodies specific for purified MPR-300.

Bovine, human and rat MPR-300 have a single high affinity binding site for IGF-II (Keiss *et al.*, 1988; MacDonald *et al.*, 1988; Nolan *et al.*, 1990; Roth *et al.*, 1987; Tong *et al.*, 1988), which also binds IGF-I with a much lower affinity (Tong *et al.*, 1988). Chicken and Xenopus MPR-300 do not bind IGF-II, however, suggesting that the acquisition of this binding site occurred in evolution after the divergence of mammals from other vertebrates (Canfield and Kornfeld, 1989; Clairmont and Czech, 1989). The IGF-II binding site is contained within repeats 5-11 of the extracytoplasmic domain, with residues in repeat 11 playing an important role (Dahms *et al.*, 1994). The M6P and IGF-II binding sites are thought not to overlap as the two ligands bind MPR-300 simultaneously (Braulke *et al.*, 1988; Waheed *et al.*, 1988), but may be close as lysosomal enzyme binding appears to sterically hinder binding of IGF-II, such that M6P enhances IGF-II binding in the presence of endogenous lysosomal enzymes (Braulke *et al.*, 1988; Keiss *et al.*, 1988; Kiess *et al.*, 1990; Polychronakos *et al.*, 1988; Schardt *et al.*, 1993; Tong *et al.*, 1988).

Whether or not the two binding sites are independent is a matter of some disagreement. Several studies have shown that neither M6P or IGF-II binding to MPR-300 influences binding of the other (Braulke *et al.*, 1988; Polychronakos and Piscina, 1988; Tong *et al.*, 1988), however Kiess *et al.* (1989) demonstrated that IGF-II inhibited binding of β-galactosidase to the surface of C6 and BRL-3A2 cells and to purified MPR-300, suggesting that binding of M6P and IGF-II are not independent.

1.11.2.6 The role of MPR-300 in IGF-II function

The insulin-like growth factors are peptides with structural homology to pro-insulin, which stimulate proliferation and differentiation in a wide range of cell types (Lund *et al.*, 1986). IGF-I is involved in metabolic processes, and has growth promoting effects on several tissues, while IGF-II is important in foetal and neonatal development (Froesch *et al.*, 1985; Rechler and Nissley, 1985). The physiological role of IGF-II binding to MPR-300 is uncertain, since it also binds to the IGF-I and insulin receptors (Morgan *et al.*, 1987), and many of its biological effects seem to be mediated by the IGF-I receptor (Roth, 1988). The major function of MPR-300 in growth regulation appears to be limiting embryonic growth by removal of extracellular IGF-II, resulting in its degradation in the lysosomes (Haig and Graham, 1991). However, a number of reports suggest that binding of IGF-II to MPR-300 may have biological significance. IGF-II binding to MPR-300 has been reported to stimulate glycogen synthesis in human HepG2 cells (Hari *et al.*, 1987); calcium influx and DNA synthesis in competent BALB/c 3T3 cells primed with EGF (Kojima *et al.*, 1988; Nishimoto *et al.*, 1987a; Nishimoto *et al.*, 1987b); proliferation of K562 cells (Tally *et al.*, 1987); motility in rhabdomyosarcoma cells (Minniti *et al.*, 1992); and formation of inositol triphosphate in the basolateral membranes of proximal kidney tubular cells (Rogers and Hammerman, 1988). In the first two studies listed, antibodies to MPR-300 were able to reproduce the effects of IGF-II (Hari *et al.*, 1987; Kojima *et al.*, 1988), and in the last, its stimulatory effect was potentiated by M6P (Rogers and Hammerman, 1989). Sakano *et al.* (1991) took a different approach and prepared two IGF-II mutants, one binding only to MPR-300 and not to the IGF-I receptor, and a second with the reverse specificity. Using these mutants, they showed that stimulation of glycogen synthesis in HepG2 cells and DNA synthesis in BALB/c 3T3 cells was induced through the IGF-I receptor, in direct contrast to the studies mentioned above. Evidently the question of whether or not MPR-300 mediates any of the biological effects of IGF-II is yet to be resolved.

1.11.2.7 MPR-300 is involved in IGF-II-mediated signal transduction

In spite of these uncertainties, MPR-300 has been shown to function in G protein activation (Figure 1.13), making a role in signal transduction very likely. In phospholipid vesicles, IGF-II stimulates direct coupling of purified MPR-300 and G_{i2}, a G protein with a 40 kDa α subunit (Murayama *et al.*, 1990; Nishimoto *et al.*, 1989). A 14 amino acid sequence from the cytoplasmic domain of MPR-300 has been identified as mediating this interaction (reviewed by Nishimoto (1993)). Activation of G_{i2} by MPR-300 can be reproduced by a synthetic peptide corresponding to this sequence, and is blocked by an antibody to this peptide, indicating a critical role for this small region of the cytoplasmic domain (Okamoto *et al.*, 1990a). The nine residues to the C-terminal end of this sequence appear to mediate binding to G_{i2}, while the N-terminal end is involved in its activation (Nishimoto, 1993). As purified MPR-300 only activates G_{i2} in the presence of IGF-II, while the isolated sequence has no such constraint, this sequence must be masked or inactivated in some way in the intact receptor, and this suppression removed by the binding of IGF-II. How extracellular IGF-II regulates the activity of this peptide is still unknown (Nishimoto, 1993).

Unlike IGF-II, neither M6P nor the lysosomal enzyme β-glucuronidase stimulates coupling of MPR-300 and G_i proteins. Thus lysosomal enzyme sorting by MPR-300 probably does not involve G_i activation (Murayama *et al.*, 1990; Okamoto *et al.*, 1990b). Conversely, both M6P and β-glucuronidase *suppress* the stimulatory effect of IGF-II on MPR-300 (Murayama *et al.*, 1990). This may account for conflicting reports concerning the signalling function of MPR-300. In several studies, MPR-300 had no effect on cellular metabolism or proliferation, even when IGF-II bound to the receptor (Conover *et al.*, 1987; Ewton *et al.*, 1987; Kiess *et al.*, 1987), while other experiments suggested that it did (Section 1.11.2.6). Such variation in receptor function may arise from the variable presence of endogenous M6P-containing proteins which counter the stimulatory effect of IGF-II (Murayama *et al.*, 1990). These investigators also speculated that inhibition of a IGF-II-mediated growth signal by M6P may be relevant to the actions of several growth modulators which contain M6P and bind MPR-300, such as the transforming growth factor β_1 precursor and proliferin. These may have the capacity to interfere with the IGF-II-mediated signalling function of MPR-300.

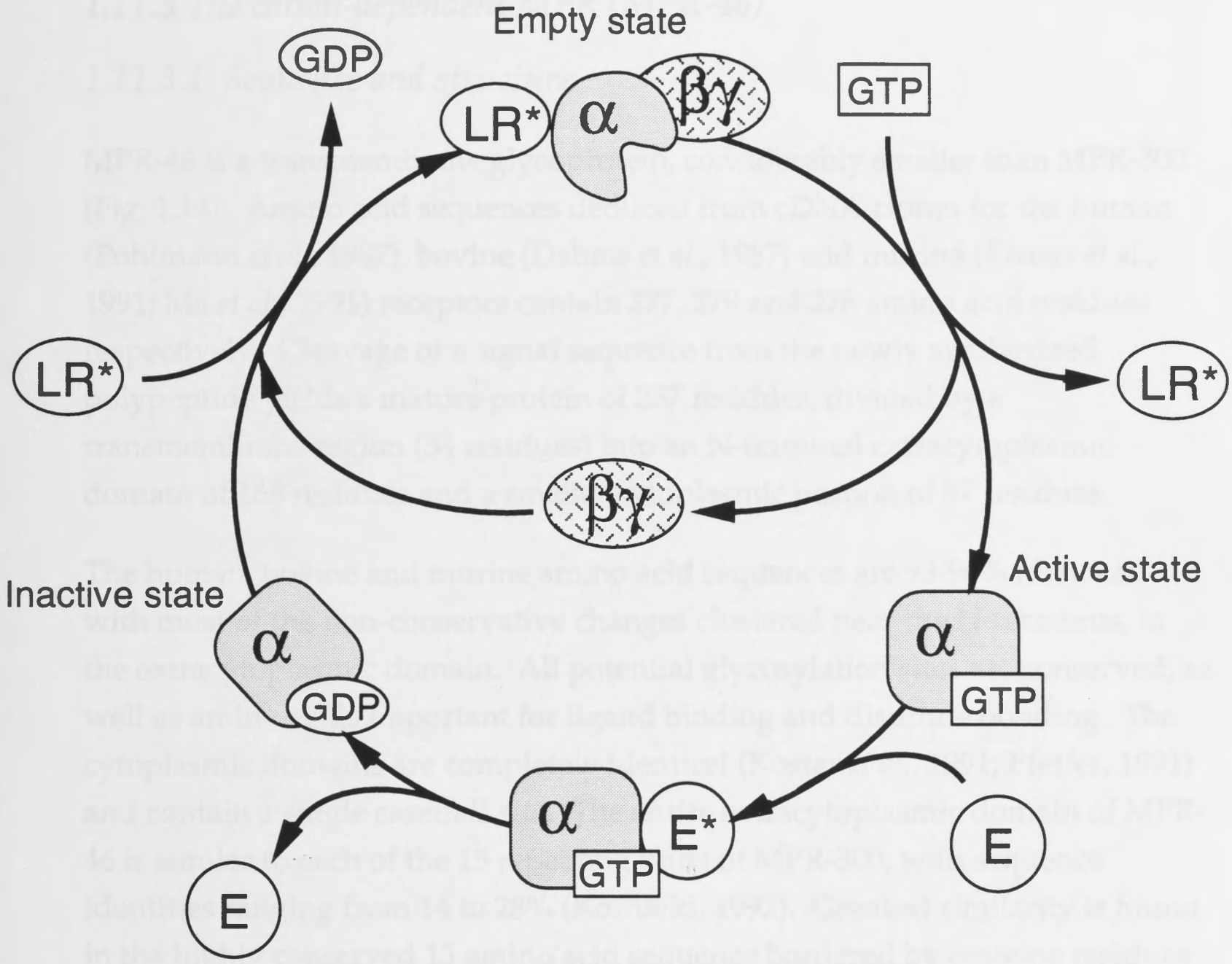


Fig. 1.13 Activation of G proteins

G proteins are a family of GTPases involved in transduction of sensory and hormonal stimuli across the plasma membrane. They mediate transmembrane signalling using a basic cycle of GTP binding and hydrolysis, whereby the GTP-bound form is "active" and the GDP-bound form "inactive" with respect to their ability to modulate the activity of effector proteins. Cell surface receptors, activated by binding of extracellular ligands (LR*), bind the GDP-bound G protein trimer and promote dissociation of GDP. GTP then binds to the α subunit, which triggers dissociation of the G protein from the receptor and separation of α.GTP from the βγ complex. The "active" GTP-bound α chain then activates an effector enzyme or ion channel (E). Hydrolysis of bound GTP by the intrinsic GTPase activity of the α chain returns it to the "inactive" state, in which it dissociates from the effector molecule and binds βγ once more. The resulting αβγ.GDP complex is then available for activation by another receptor molecule (Bourne *et al.*, 1990).

1.11.3 The cation-dependent MPR (MPR-46)

1.11.3.1 Sequence and structure

MPR-46 is a transmembrane glycoprotein, considerably smaller than MPR-300 (Fig. 1.14). Amino acid sequences deduced from cDNA clones for the human (Pohlmann *et al.*, 1987), bovine (Dahms *et al.*, 1987) and murine (Koster *et al.*, 1991; Ma *et al.*, 1991) receptors contain 277, 279 and 278 amino acid residues respectively. Cleavage of a signal sequence from the newly synthesized polypeptide yields a mature protein of 257 residues, divided by a transmembrane region (34 residues) into an N-terminal extracytoplasmic domain of 165 residues and a smaller cytoplasmic portion of 67 residues.

The human, bovine and murine amino acid sequences are 93-96% identical, with most of the non-conservative changes clustered near the N-terminus, in the extracytoplasmic domain. All potential glycosylation sites are conserved, as well as amino acids important for ligand binding and disulfide bonding. The cytoplasmic domains are completely identical (Koster *et al.*, 1991; Pfeffer, 1991) and contain a single casein II site. The entire extracytoplasmic domain of MPR-46 is similar to each of the 15 repeating units of MPR-300, with sequence identities ranging from 14 to 28% (Kornfeld, 1992). Greatest similarity is found in the highly conserved 13 amino acid sequence bordered by cysteine residues which is found in all the sequence repeats of MPR-300 (Dahms *et al.*, 1987). Such sequence homology suggests a common ancestral gene for the extracytoplasmic domains of the two receptors, with MPR-300 arising through multiple duplications (Kornfeld, 1992). As the sequence encoding this domain spans four exons in the MPR-46 gene, duplication of the putative ancestral gene must have occurred before the introduction of the present intron sequences (Klier *et al.*, 1991). There is no homology between the signal sequences and transmembrane regions of MPR-300 and MPR-46 (Kornfeld, 1992), and very little between the cytoplasmic domains, which is notable in view of the similar intracellular movement of these receptors. The only feature they share with one another, and with other endocytic receptors, is that all contain cysteine residues and clusters of acidic residues (Dahms *et al.*, 1987).

1.11.3.2 Glycosylation

MPR-46 is heavily glycosylated, appearing as a diffuse band on SDS-PAGE gels. The deglycosylated form migrates with an apparent molecular mass of 26.5-28 kDa by SDS-PAGE, corresponding to the calculated mass of 28-29 kDa

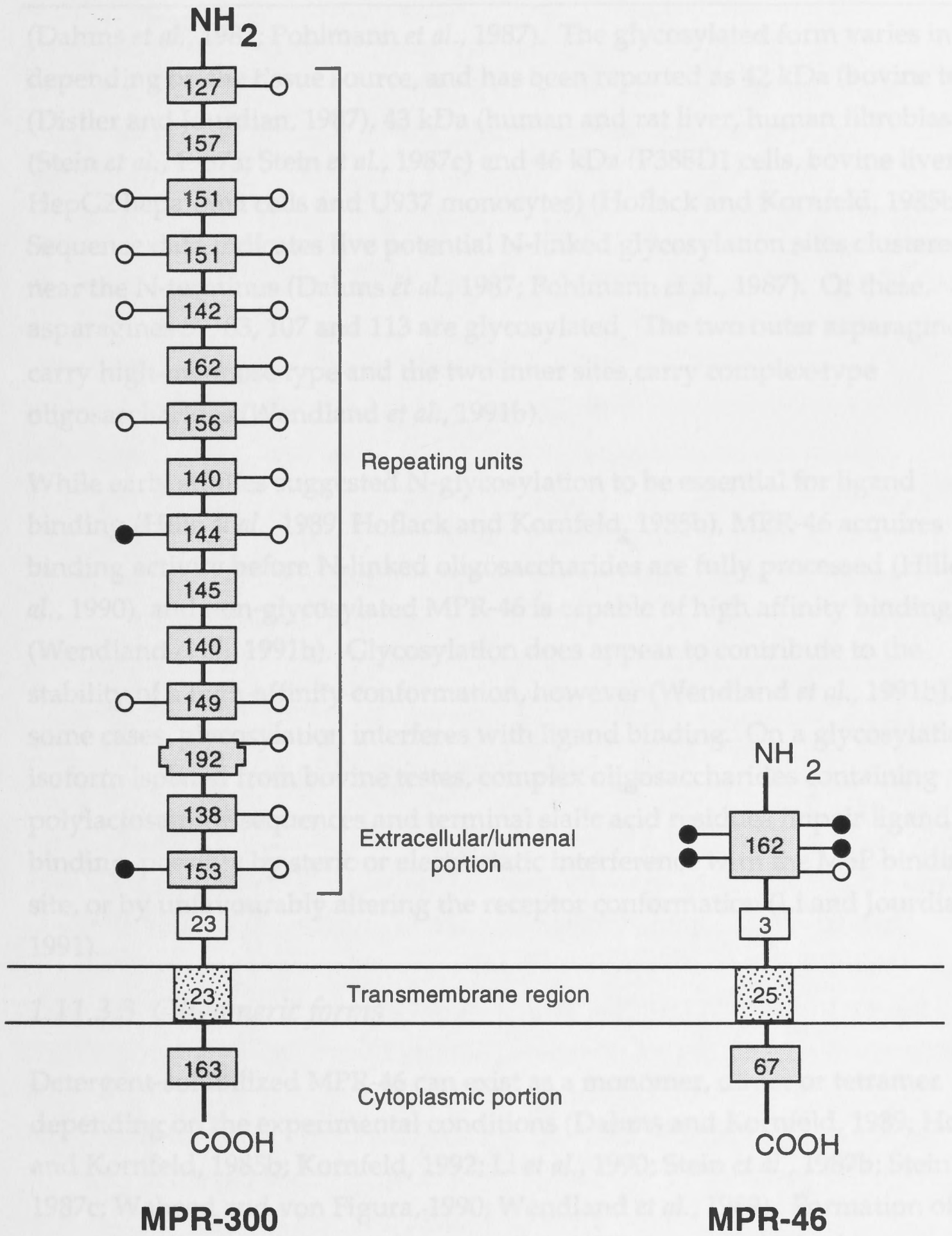


Fig. 1.14 Schematic representation of MPR-46: comparison with MPR-300.

Segmentation of the extracellular portions into domains is based on optimal alignment of repeating units. The number of amino acid residues in each domain is shown. Closed circles represent N-linked glycosylation sites known to be used, while open circles represent potential glycosylation sites

Modified from Kornfeld (1987).

(Dahms *et al.*, 1987; Pohlmann *et al.*, 1987). The glycosylated form varies in size depending on the tissue source, and has been reported as 42 kDa (bovine testes) (Distler and Jourdian, 1987), 43 kDa (human and rat liver, human fibroblasts) (Stein *et al.*, 1987a; Stein *et al.*, 1987c) and 46 kDa (P388D1 cells, bovine liver, HepG2 hepatoma cells and U937 monocytes) (Hoflack and Kornfeld, 1985b). Sequence data indicates five potential N-linked glycosylation sites clustered near the N-terminus (Dahms *et al.*, 1987; Pohlmann *et al.*, 1987). Of these, asparagines 57, 83, 107 and 113 are glycosylated. The two outer asparagines carry high-mannose type and the two inner sites carry complex-type oligosaccharides (Wendland *et al.*, 1991b).

While early studies suggested N-glycosylation to be essential for ligand binding (Hille *et al.*, 1989; Hoflack and Kornfeld, 1985b), MPR-46 acquires binding activity before N-linked oligosaccharides are fully processed (Hille *et al.*, 1990), and non-glycosylated MPR-46 is capable of high affinity binding (Wendland *et al.*, 1991b). Glycosylation does appear to contribute to the stability of a high-affinity conformation, however (Wendland *et al.*, 1991b). In some cases, glycosylation interferes with ligand binding. On a glycosylation isoform isolated from bovine testes, complex oligosaccharides containing polylactosamine sequences and terminal sialic acid residues impair ligand binding, possibly by steric or electrostatic interference with the M6P binding site, or by unfavourably altering the receptor conformation (Li and Jourdian, 1991).

1.11.3.3 Oligomeric forms

Detergent-solubilized MPR-46 can exist as a monomer, dimer or tetramer depending on the experimental conditions (Dahms and Kornfeld, 1989; Hoflack and Kornfeld, 1985b; Kornfeld, 1992; Li *et al.*, 1990; Stein *et al.*, 1987b; Stein *et al.*, 1987c; Waheed and von Figura, 1990; Wendland *et al.*, 1989). Formation of the tetramer is favoured at low temperature (<16°C), neutral pH, high receptor concentration and in the presence of M6P, whereas dissociation is favoured by higher temperatures, low pH and low receptor concentration. The three forms rapidly interconvert at 37°C (Waheed and von Figura, 1990; Waheed *et al.*, 1990), and the subunits interact by non-covalent bonds, as they can be dissociated by SDS under non-reducing conditions (Hoflack and Kornfeld, 1985b). The transmembrane domain appears to be involved in oligomerization, as a series of truncated MPR-46 constructs showed that the extracytoplasmic domain alone is insufficient for aggregation, whereas the cytoplasmic domain is unnecessary (Dahms and Kornfeld, 1989). Oligomers may be partially

stabilized by interactions between extracytoplasmic domains, however, as a similar MPR-46 construct, containing the extracytoplasmic domain and the first amino acid of the transmembrane region, was able to form dimers (Wendland *et al.*, 1989).

Several cross-linking studies have shown that MPR-46 exists in membranes as non-covalently linked homodimers (Dahms and Kornfeld, 1989; Li *et al.*, 1990; Stein *et al.*, 1987a; Stein *et al.*, 1987b). However, monomeric, dimeric and tetrameric forms have all been observed in BHK cells overexpressing the receptor (Waheed *et al.*, 1990). As the equilibrium between oligomeric forms is influenced by pH, and ligand and receptor concentrations in a manner that would favour MPR-46 association in the Golgi/TGN, where it binds ligand at near neutral pH, and dissociation in the acidic prelysosomal compartment where ligand is released, these authors have speculated that the quaternary structure of MPR-46 may change during its cycling between the different compartments, and may even contribute to the trafficking of the receptor (Waheed and von Figura, 1990).

1.11.3.4 Binding of phosphomannosyl ligands

MPR-46 is specific for oligosaccharides bearing terminal M6P residues (Pfeffer, 1991). As with MPR-300, binding is enhanced when M6P is linked α1,2 to a mannose residue, suggesting that the binding site may accomodate an extended oligosaccharide structure which includes the M6P α-1,2 man sequence (Tong and Kornfeld, 1989). Unlike MPR-300, MPR-46 does not bind the methylphosphomannosyl groups present on lysosomal enzymes from the slime mold *Dictyostelium discoideum* (Hoflack and Kornfeld, 1985a). MPR-46 has a narrower pH optimum than MPR-300 (pH 6-6.3); ligand binding is reduced as the pH becomes neutral, and is non-existent below pH 5.3 (Hoflack *et al.*, 1987; Tong and Kornfeld, 1989).

Monomeric MPR-46 is capable of binding phosphomannosyl ligands (Dahms and Kornfeld, 1989; Wendland *et al.*, 1989). Its affinity for M6P is low, however, and requires the presence of Mn^{2+} (Li *et al.*, 1990), in fact Waheed and von Figura (1990) could not detect binding of the monomer to a phosphomannan-Sepharose affinity column. Ligand binding is enhanced with increasing oligomerization (Li *et al.*, 1990; Waheed *et al.*, 1990), possibly due to changes in receptor conformation which make the binding site more accessible, or the ability of oligomers to bind multivalent ligands (Li *et al.*, 1990). MPR-46 binds one mole of M6P, and 0.5 mole of a diphosphorylated high-mannose

oligosaccharide per monomeric subunit, thus each dimer has two binding sites, both of which can be occupied by a divalent ligand (Distler *et al.*, 1991; Tong and Kornfeld, 1989). As a consequence, dimeric MPR-46 has a higher affinity for multivalent ligands such as lysosomal enzymes than for monovalent ligands (Hoflack *et al.*, 1987; Ma *et al.*, 1991; Tong and Kornfeld, 1989).

The M6P binding site resides on the extracytoplasmic domain of MPR-46, as lysosomal enzyme binding occurs on the luminal side of the membrane. His-131 and Arg-137 are essential for ligand binding, as conservative replacement of these two residues by site-directed mutagenesis results in loss of binding activity without affecting other properties of the receptor. Chemical modification of arginine and histidine residues also destroys M6P binding (Wendland *et al.*, 1991a). The presence of M6P during modification of arginine, but not histidine, protects against loss of binding activity, suggesting that arg-137 is a component of the M6P binding site while his-131 may influence binding in a more indirect manner (Stein *et al.*, 1987c). From the perspective of the ligand, the 6-phosphate and 2-hydroxyl groups of M6P both contribute to the binding interaction, probably through ionic or hydrogen bonding for the former, and hydrogen bonding for the latter (Tong and Kornfeld, 1989). The importance of ionic bonding suggests the presence of cationic amino acid residues in the binding pocket, consistent with the involvement of arg-137. Hydrogen bonding in protein-sugar interactions is mediated by the planar side chains of such amino acids as arginine, aspartic acid, glutamic acid and asparagine (Quiocho, 1986), which are abundant in the conserved membrane proximal region of the MPR-46 extracytoplasmic domain (Pfeffer, 1991).

1.11.3.5 Requirement for divalent cations

Although MPR-46 is frequently referred to as the cation-dependent MPR, the bovine and murine forms appear more reliant on divalent cations (Distler *et al.*, 1987; Hoflack *et al.*, 1987; Hoflack and Kornfeld, 1985a; Hoflack and Kornfeld, 1985b; Li *et al.*, 1990; Ma *et al.*, 1991) than are human or porcine MPR-46 (Baba *et al.*, 1988; Junghans *et al.*, 1988; Watanabe *et al.*, 1990). However, although Mn^{2+} enhances binding to bovine MPR-46 at low ligand concentrations, it has little effect as the receptor approaches saturation, suggesting that divalent cations serve to increase the association constant (K_a) of the receptor-ligand complex rather than being an absolute requirement for binding (Distler *et al.*, 1987). Divalent cations also have a greater effect on the dimeric and monomeric forms of MPR-46 than on the tetramer, which may explain the apparent species-specific requirements for divalent cations, as the receptor preparations used in

the various studies may have contained differing proportions of the various oligomeric forms (Li *et al.*, 1990).

1.12 Intracellular transport of MPR-enzyme complexes

1.12.1 Subcellular localization of MPR-300 and MPR-46

MPR-300 and MPR-46 have a predominantly intracellular location. MPR-300 was initially localized in subcellular fractions of rat liver by Fischer *et al.* (1980b), who reported 90% in the fraction containing ER, Golgi and lysosomal membranes, 9.5% in the plasma membrane and less than 1% in the nuclear/mitochondrial fraction. Subsequent immunocytochemical and biochemical studies have shown that at steady-state, most MPR-300 is present in one or more populations of endosomes, with smaller amounts in the Golgi apparatus, on the cell surface and in coated vesicles, and little or none in the lysosomes (Brown and Farquhar, 1984a; Brown and Farquhar, 1984b; Brown *et al.*, 1986; Geuze *et al.*, 1984a; Geuze *et al.*, 1985; Geuze *et al.*, 1984b; Geuze *et al.*, 1988; Goda and Pfeffer, 1988; Griffiths *et al.*, 1988; Griffiths *et al.*, 1990; Jin *et al.*, 1989; Messner *et al.*, 1989; Willingham *et al.*, 1983; Willingham *et al.*, 1981). MPR-300 and MPR-46 are expressed simultaneously in most cells and have a similar subcellular distribution (Bleekemolen *et al.*, 1988; Gabel *et al.*, 1983). MPR-46 is localized primarily in the endosomes of U937 monocytes, mouse macrophages and proximal tubule cells (Bleekemolen *et al.*, 1988), and cycles through *trans* Golgi elements containing sialyltransferase in CHO cells (Duncan and Kornfeld, 1988). Membrane preparations isolated by antibodies specific to each receptor are not identical, however, which may reflect small differences in their cellular distribution (Messner *et al.*, 1989).

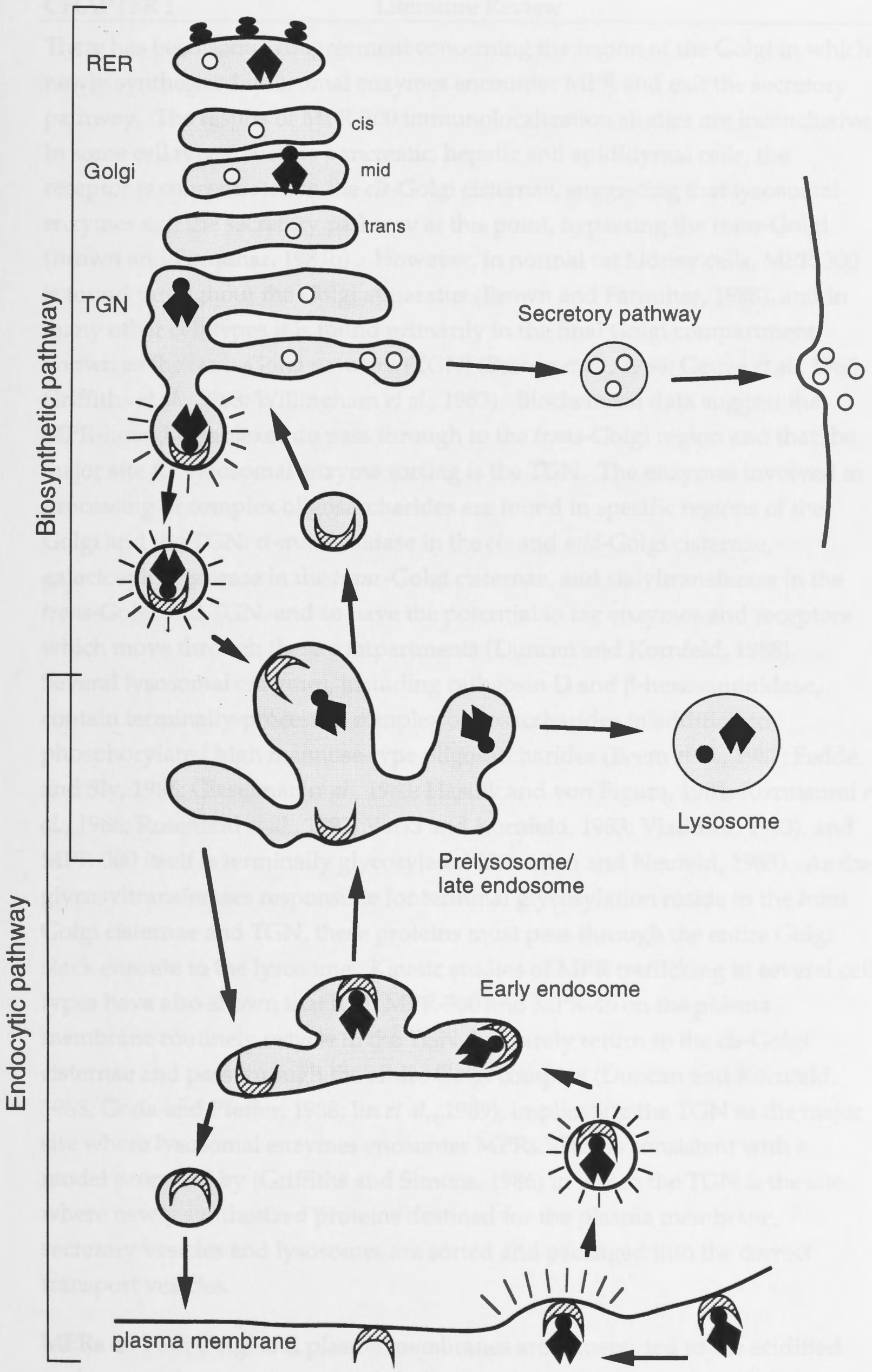
1.12.2 Intracellular movement of MPR-300 and MPR-46

As outlined in Section 1.9, MPRs mediate the targeting of lysosomal enzymes to lysosomes via both a biosynthetic and an endocytic pathway (Fig. 1.15). In the biosynthetic pathway, newly synthesized enzymes are bound by MPRs in the Golgi membranes and transported to an acidified prelysosomal organelle. Extracellular enzymes are captured by cell surface MPRs, and delivered by receptor-mediated endocytosis to the same acidic compartment, where the low pH causes M6P-containing ligands to dissociate. The enzymes later appear in mature lysosomes, while MPRs recycle back to the Golgi or the cell surface for another round of transport (reviewed by Duncan and Kornfeld (1988) and Jin *et al.* (1989)).

Fig. 1.15 Schematic diagram of lysosomal enzyme targeting to lysosomes

Lysosomal enzymes (◆) and secretory proteins (O) are synthesized in the rough ER and transported to the Golgi, where lysososomal enzymes acquire M6P markers (◆●). Most lysosomal enzymes bind MPRs () in the trans Golgi network (TGN) and are transported in coated vesicles to an acidified prelysosomal/endosomal compartment, where they are released. The enzymes are packaged into lysosomes where the M6P marker is inactivated by dephosphorylation. The receptors return to the Golgi or the plasma membrane for another round of lysosomal enzyme transport.

Extracellular lysosomal enzymes may be delivered to the lysosomes via the endocytic pathway. A small portion of enzymes is typically secreted by cells, and these may bind to cell surface MPR and be internalized via clathrin-coated pits and vesicles. The enzymes are delivered to the acidifiedendosomal compartment, where they dissociate from MPRs and are delivered to the lysosomes.


RER
cis
Golgi
mid
trans
TGN
Biosynthetic pathway
Secretory pathway
Lysosome
Prelysosome/
late endosome
Early endosome
Endocytic pathway
plasma membrane

There has been some disagreement concerning the region of the Golgi in which newly synthesized lysosomal enzymes encounter MPR and exit the secretory pathway. The results of MPR-300 immunolocalization studies are inconclusive. In some cell types, such as pancreatic, hepatic and epididymal cells, the receptor is concentrated in the *cis*-Golgi cisternae, suggesting that lysosomal enzymes exit the secretory pathway at this point, bypassing the *trans*-Golgi (Brown and Farquhar, 1984b) . However, in normal rat kidney cells, MPR-300 is found throughout the Golgi apparatus (Brown and Farquhar, 1988), and in many other cell types it is found primarily in the final Golgi compartment, known as the *trans*-Golgi network (TGN) (Brown *et al.*, 1986; Geuze *et al.*, 1985; Griffiths *et al.*, 1988; Willingham *et al.*, 1983). Biochemical data suggest that MPR-ligand complexes do pass through to the *trans*-Golgi region and that the major site for lysosomal enzyme sorting is the TGN. The enzymes involved in processing of complex oligosaccharides are found in specific regions of the Golgi and the TGN: α-mannosidase in the *cis* and *mid*-Golgi cisternae, galactosyltransferase in the *trans*-Golgi cisternae, and sialyltransferase in the *trans*-Golgi and TGN, and so have the potential to tag enzymes and receptors which move through these compartments (Duncan and Kornfeld, 1988). Several lysosomal enzymes, including cathepsin D and β-hexosaminidase, contain terminally-processed complex oligosaccharides in addition to phosphorylated high mannose-type oligosaccharides (Beem *et al.*, 1987; Fedde and Sly, 1985; Gieselman *et al.*, 1983; Hasilik and von Figura, 1981; Kozutsumi *et al.*, 1986; Rosenfeld *et al.*, 1982; Varki and Kornfeld, 1983; Vladutiu, 1983), and MPR-300 itself is terminally glycosylated (Sahagian and Neufeld, 1983). As the glycosyltransferases responsible for terminal glycosylation reside in the *trans* Golgi cisternae and TGN, these proteins must pass through the entire Golgi stack enroute to the lysosome. Kinetic studies of MPR trafficking in several cell types have also shown that both MPR-300 and MPR-46 on the plasma membrane routinely recycle to the TGN, but rarely return to the *cis*-Golgi cisternae and pass through the entire Golgi complex (Duncan and Kornfeld, 1988; Goda and Pfeffer, 1988; Jin *et al.*, 1989), implicating the TGN as the major site where lysosomal enzymes encounter MPRs. This is consistent with a model proposed by (Griffiths and Simons, 1986) in which the TGN is the site where newly synthesized proteins destined for the plasma membrane, secretory vesicles and lysosomes are sorted and packaged into the correct transport vesicles.

MPRs on both Golgi and plasma membranes are transported to the acidified prelysosomal compartment in clathrin-coated vesicles (Pearse and Robinson,

1990). MPR-300 has been immunolocalized to coated vesicles near the Golgi stack in fibroblasts (Brown and Farquhar, 1984a), and lysosomal enzyme precursors transiently associate with coated membranes and coated vesicles in a M6P-dependent manner (Lemansky *et al.*, 1987; Schulze-Lohoff *et al.*, 1985). The transport process is best understood in the context of endocytosis. Cell surface receptors requiring internalization are found clustered in clathrin-coated pits, which exclude other membrane proteins, thus separating proteins that are to be transported to another compartment from those that are to remain behind (Pearce and Bretscher, 1981). Coated pits bud off into the cytoplasm as coated vesicles, the coats disassemble, and the vesicles, with their cargo of receptors, fuse with a second compartment. This process allows specific receptors to bind soluble proteins on one membrane and deliver them efficiently to another compartment (reviewed by Pearse and Robinson (1990)). Transport of MPRs in coated vesicles appears to be an energy-dependent process, as GTP hydrolysis is required for vesicular transport of MPR-300 from the the prelysosomal compartment to the TGN in semi-intact cell extracts (Goda and Pfeffer, 1988). The rab family of ras-like GTPases may play a key role in regulating vesicular transport, for example, rab9 is essential for MPR-300 recycling from the prelysosomal compartment to the TGN (Lombardi *et al.*, 1993; Riederer *et al.*, 1994), rab5 regulates early endosome fusion, rab4 functions in receptor recycling between early endosomes and the plasma membrane, and rab1 is involved in protein transport between the ER and the Golgi. These proteins are believed to function in ensuring the accuracy of vesicle targeting and fusion events (reviewed by Bourne *et al.* (1990), Lombardi *et al.* (1993), and Riederer *et al.* (1994)).

MPR-enzyme complexes are delivered to an acidic reticular-vesicular structure close to the Golgi apparatus (Griffiths *et al.*, 1988). Several studies suggest that this is identical to the endocytic CURL (Compartment of Uncoupling Receptors and Ligands) (Brown *et al.*, 1986; Geuze *et al.*, 1984a; Geuze *et al.*, 1985; Geuze *et al.*, 1984b; Pfeffer, 1987), an organelle intermediate between clathrin-coated endocytic vesicles and lysosomes, in which dissociation of receptor-ligand complexes appears to occur (Geuze *et al.*, 1983). This prelysosomal/endocytic compartment has been characterized in NRK cells and has a complex three-dimensional structure, consisting of tubuol-reticular domains in continuity with vesicular parts. The latter contain internal membranes, either tubular or sheet-like, which are rich in MPR-300. Lysosomes may form from this compartment either by budding off or somehow "maturing" (Brown *et al.*, 1986; Griffiths *et al.*, 1990).

1.12.3 Intracellular and cell surface MPRs belong to a common pool

Considerable evidence demonstrates that the cell surface and intracellular MPR pools are in equilibrium. Addition of MPR-300 antibody to the culture medium of fibroblasts inhibited both endocytosis of extracellular enzymes and sorting of newly synthesized ones, indicating that intracellular receptors were accessible to the antibody. Exposure of most of the MPR-300 pool to extracellular antibody implied a constant exchange between intracellular and cell surface receptors (Gartung *et al.*, 1985; Nolan, 1987; Sahagian, 1984; von Figura *et al.*, 1984). One objection to this interpretation was raised by Kornfeld and Mellman (1989), who suggested that since these experiments were performed at 37°C, the antibody may have been internalized by fluid phase endocytosis and so contacted the intracellular receptor pool directly.

More convincing evidence has been provided by biochemical studies showing that MPR-300 can be modified by Golgi-specific enzymes after expression on the cell surface. Cell surface MPR-300 and MPR-46 labelled with [^{3}H] galactose at 4°C were rapidly sialylated when the cells were warmed to 37°C. As sialyltransferase is located in the *trans*-Golgi and TGN, this demonstrated that cell surface MPRs routinely reach the Golgi apparatus after endocytosis (Duncan and Kornfeld, 1988). In a similar study, cell surface MPR-300 was desialylated by neuraminidase treatment. The receptors were rapidly resialylated at 37°C, indicating again that surface MPR-300 is transported to the *trans*- Golgi region. As only a fraction of the resialylated receptor returned to the cell surface, this also suggested that the cell surface and intracellular MPR pools were mixed after resialylation, and that a single pool of receptors cycles between all MPR-containing compartments (Jin *et al.*, 1989).

1.12.4 Control of MPR movement

1.12.4.1. Effects of ligand binding

Several investigators have proposed that MPR movement is regulated by ligand occupancy. (Brown *et al.*, 1984) showed that when Clone 9 hepatocytes were treated with tunicamycin, an inhibitor of N-linked glycosylation which prevents formation of the M6P marker on newly synthesized lysosomal enzymes, MPR-300 accumulated in the *cis*-Golgi at the expense of the prelysosomal compartment. Conversely, when ligand dissociation in the prelysosomal/endosomal compartment was prevented by raising the pH with weak bases (chloroquine or ammonium chloride), MPR-300 accumulated in the

endosomes and was depleted from the Golgi. Recycling to the Golgi was re-established by allowing the cells to endocytose M6P, facilitating MPR-enzyme dissociation inspite of the high pH (Brown *et al.*, 1986). These studies suggested that ligand binding triggers movement of MPR-300 to endosomes, and ligand dissociation triggers their return to the Golgi. Similarly, MPR-300 accumulated in the Golgi and in nearby coated vesicles, and was depleted from endosomes in I-cell fibroblasts, in which lysosomal enzymes lack the M6P recognition marker (Brown and Farquhar, 1984a). MPR-300 was thus enriched at the sorting site and reduced or absent at the delivery site. These investigators concluded that coated vesicles containing MPR-300 do not constitutively move along the normal delivery route from Golgi to lysosomes, but are triggered to do so by enzyme binding.

In contrast, others have found that MPR-300 recycling through the biosynthetic pathway is constitutive. When HepG2 cells were treated with cycloheximide or tunicamycin, producing a deficiency of MPR ligands in the Golgi, endosomal MPR-300 was not reduced, suggesting that in this case it's movement along the intracellular route was independent of ligand binding (Pfeffer, 1987). Intracellular sorting of lysosomal enzymes is also unaffected by overexpression of IGF-II, indicating that binding of this ligand does not influence the cellular distribution of MPR-300 (Braulke *et al.*, 1991).

The distribution of MPR-300 between the cell surface and the endosomal compartment appears to be influenced by receptor occupancy, however movement between these two compartments is not. Preventing ligand release in the acidified prelysosomal compartment with weak bases reduced cell surface MPR by 20-50% (Braulke *et al.*, 1987; Braulke *et al.*, 1992; Geuze *et al.*, 1985; Gonzalez-Noriega *et al.*, 1980), but the rate of exchange between internal and cell surface MPR-300 was not affected. Free cell surface receptors were replaced with occupied ones, which continued to exchange with intracellular MPR-ligand complexes at the normal rate (Braulke *et al.*, 1987). Extracellular M6P-containing ligands also had no effect on the internalization of cell surface MPR-300, as determined by resialylation of desialylated cell surface MPR-300 in the TGN (Jin *et al.*, 1989), nor did extracellular IGF-II (Oka and Czech, 1986). These studies suggest that receptor occupancy does not influence the exchange of MPR-300 between the cell surface and internal membranes, in either direction. This exchange was also unaffected by depletion of newly synthesized lysosomal enzymes from the Golgi by cycloheximide treatment, which indirectly implies that movement of receptors between internal

compartments is also constitutive (Braulke *et al.*, 1987; Jin *et al.*, 1989). These studies suggest that the MPRs move constantly between the Golgi or plasma membrane and the prelysosomal compartment, and transport lysosomal enzymes by virtue of the pH difference between these two compartments. The approximately neutral pH in the Golgi or at the cell surface permits the binding of M6P-bearing ligands, while the acidic conditions in the prelysosomal compartment favour their release.

1.12.4.2. The role of the cytoplasmic domain in MPR movement

The cytoplasmic domains of both MPR-300 and MPR-46 regulate their movement between intracellular compartments. By transfecting MPR-300 mutants with deletions in the cytoplasmic tail into receptor-deficient cells, Lobel *et al.* (1989) showed the C-terminal region to be important for intracellular trafficking, and the membrane-proximal portion essential for endocytosis (Lobel *et al.*, 1989). The cytoplasmic tail of MPR-46, completely conserved between species (Koster *et al.*, 1991), is also essential for internalization, as truncated receptors lacking this domain accumulate at the cell surface (Johnson *et al.*, 1990; Peters *et al.*, 1990; Weber *et al.*, 1989). Signals required for MPR-46 cycling between the Golgi apparatus and prelysosomal compartment may also reside in the cytoplasmic tail (Nadimpalli *et al.*, 1991).

The cytoplasmic tails appear to include recognition sequences which target the receptors for inclusion in coated pits on the Golgi and plasma membranes, and hence for packaging into coated vesicles. Coated pits are associated with two distinct structural units: the clathrin triskelion and an adaptor protein. The adaptors interact with a recognition sequence in the cytoplasmic tail of appropriate receptors, gathering them into the coated pits while excluding other membrane proteins. Clathrin interacts with the adaptors, forming an outer polyhedral cage around the pit or vesicle (Fig. 1.16). Two adaptors, HA-I and HA-II, have been identified and are found in coated pits on the Golgi and plasma membranes respectively (Pearse and Robinson, 1990). They appear to recognise distinct amino acid sequences, such that the LDL and transferrin receptors associate with HA-II at the plasma membrane and are constitutively endocytosed, while newly synthesized receptors fail to interact with HA-I in the Golgi (Pearse and Robinson, 1990).

Both adaptors bind to distinct regions of the MPR cytoplasmic tail, thus MPRs associate with HA-II adaptors in plasma membrane coated pits, and with HA-I adaptors in the Golgi (Glickman *et al.*, 1989; Pearse and Robinson, 1990). The

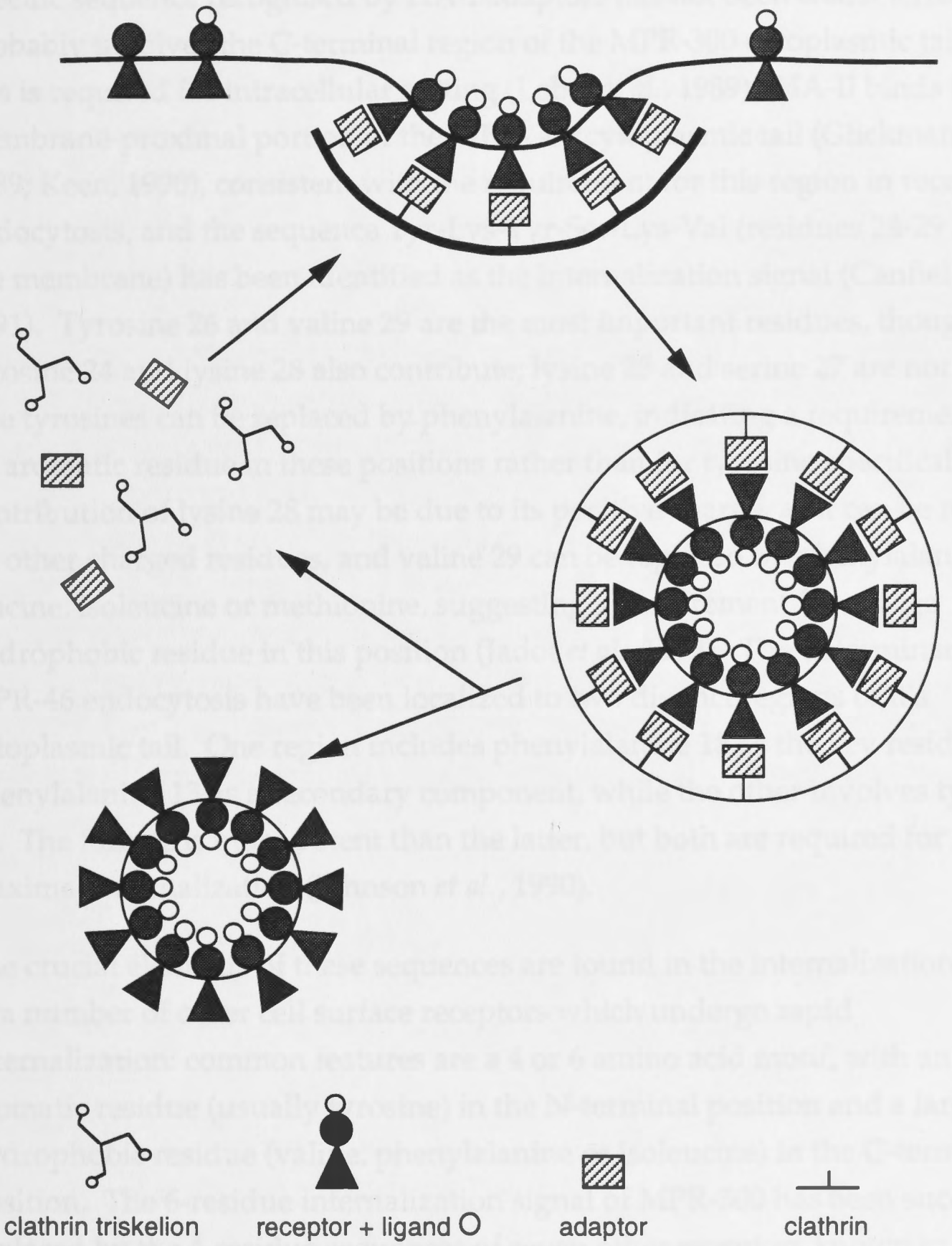


Fig. 1.16 General scheme of a cycle of coated pit formation

Soluble clathrin triskelions and adaptors interact at the membrane with the cytoplasmic tails of receptors. Coated pits form by assembly of further receptors and coat components, and pinch off to form coated vesicles. These rapidly uncoat, releasing the vesicle and allowing the soluble coat components to form another coated pit.

Adapted from Pearse and Robinson (1990)

specific sequence recognised by HA-I adaptors has not been characterized, but probably involves the C-terminal region of the MPR-300 cytoplasmic tail, as this is required for intracellular sorting (Lobel *et al.*, 1989). HA-II binds to the membrane-proximal portion of the MPR-300 cytoplasmic tail (Glickman *et al.*, 1989; Keen, 1990), consistent with the requirement for this region in receptor endocytosis, and the sequence Tyr-Lys-Tyr-Ser-Lys-Val (residues 24-29 from the membrane) has been identified as the internalization signal (Canfield *et al.*, 1991). Tyrosine 26 and valine 29 are the most important residues, though tyrosine 24 and lysine 28 also contribute; lysine 25 and serine 27 are not critical. The tyrosines can be replaced by phenylalanine, indicating a requirement for an aromatic residue in these positions rather than for tyrosine specifically. The contribution of lysine 28 may be due to its positive charge, as it can be replaced by other charged residues, and valine 29 can be replaced by phenylalanine, leucine, isoleucine or methionine, suggesting a requirement for a large hydrophobic residue in this position (Jadot *et al.*, 1992). The determinants for MPR-46 endocytosis have been localized to two distinct regions of it's cytoplasmic tail. One region includes phenylalanine 18 as the key residue with phenylalanine 13 as a secondary component, while the other involves tyrosine 45. The former is more potent than the latter, but both are required for maximal internalization (Johnson *et al.*, 1990).

The crucial elements of these sequences are found in the internalization signals of a number of other cell surface receptors which undergo rapid internalization: common features are a 4 or 6 amino acid motif, with an aromatic residue (usually tyrosine) in the N-terminal position and a large hydrophobic residue (valine, phenylalanine or isoleucine) in the C-terminal position. The 6-residue internalization signal of MPR-300 has been successfully replaced by the 4-residue sequences of seven other receptors known to undergo rapid internalization. The signal for receptor internalization is thus a general motif, interchangeable between receptors, rather than a specific sequence (Jadot *et al.*, 1992).

1.12.4.3 Other factors influencing MPR movement

While the cytoplasmic tails of MPR-300 and MPR-46 are clearly involved in regulating intracellular movement, the extracytoplasmic and transmembrane domains may also have some influence. A chimeric protein comprising the extracellular and transmembrane domains of the human EGF receptor, joined to the MPR-300 cytoplasmic domain, was found predominantly at the cell surface, whereas most MPR-300 is intracellular. However, chimeric receptors

comprised of MPR-300 extracellular and transmembrane sequences joined to the cytoplasmic domains of the EGF or LDL receptors were efficiently endocytosed, and colocalized with native MPR-300 in intracellular compartments. These experiments suggest that the extracytoplasmic or transmembrane domains of MPR-300 contain an "endosome-retention" signal which keeps the native receptor in the endosomal compartment (Dintzis and Pfeffer, 1990; Dintzis *et al.*, 1994).

The quaternary structure of MPR-46 may also regulate receptor trafficking. The equilibrium between the monomeric, dimeric and tetrameric forms of the receptor is controlled by pH, ligand binding and receptor concentration, all of which are subject to variation between the sites where MPR-46 binds and releases lysosomal enzymes (Waheed and von Figura, 1990; Waheed *et al.*, 1990) (Section 1.11.3.3). This has led to speculation that the quaternary structure of MPR-46 not only varies in different cellular compartments, but actively contributes to receptor trafficking. As the conformation of the cytoplasmic tail is affected by the quaternary structure of the receptor, changes in quaternary structure may expose signals required for intracellular movement (Nadimpalli *et al.*, 1991).

1.13 Comparison of the roles of MPR-300 and MPR-46 in lysosomal enzyme targeting

1.13.1 Both MPR-300 and MPR-46 transport newly synthesized lysosomal enzymes

Both MPR-300 and MPR-46 have been implicated in the binding and targeting of newly synthesized M6P-bearing proteins to lysosomes. MPR-300 is predominantly involved in the intracellular retention of lysosomal enzymes, in that it transports newly synthesized enzymes to the prelysosomal compartment, and internalizes extracellular enzymes. Thus, cells which lack MPR-300 (Gabel *et al.*, 1983), or are depleted of it by specific antibodies (von Figura *et al.*, 1984), secrete newly synthesized enzymes in large amounts and are unable to endocytose extracellular ligands. Transfection of MPR-300 cDNA into MPR-300-deficient cell lines almost completely corrects the hypersecretion defect and allows endocytosis of exogenous ligands (Kyle *et al.*, 1988; Lobel *et al.*, 1989).

MPR-46 also participates in targeting of newly synthesized enzymes to the lysosomes. Although cells deficient in MPR-300 hypersecrete acid hydrolases,

30-40% of newly synthesized enzymes are still directed to lysosomes (Gabel *et al.*, 1983), and immunodepletion of MPR-46 increases enzyme secretion further (Stein *et al.*, 1987d). Transgenic mice in which the MPR-46 gene is deleted, but MPR-300 is retained, exhibit defects in lysosomal enzyme targeting: elevated levels of phosphorylated lysosomal enzymes are present in the body fluids, and are secreted from cultured cells (Koster *et al.*, 1993; Ludwig *et al.*, 1993).

Both receptors appear to be required for optimal intracellular sorting of lysosomal enzymes. *In vitro* studies have suggested this not to be the case, as immunodepletion of MPR-300 in cells expressing both receptors increases lysosomal enzyme secretion, while depletion of MPR-46 does not, suggesting that both receptors can participate in sorting, but MPR-300 dominates when both are present (Braulke *et al.*, 1989; Nolan, 1987; Stein *et al.*, 1987d). In contrast, the studies using MPR-46-deficient transgenic mice clearly show that a complete loss of MPR-46 is associated with misrouting of up to 50% of M6P-containing polypeptides. MPR-300 thus cannot compensate for the complete absence of MPR-46, which appears to be responsible for half of the lysosomal enzyme targeting (Ludwig *et al.*, 1993).

1.13.2 MPR-46 is not involved in endocytosis of lysosomal enzymes

Although MPR-46 contributes to sorting of newly synthesized lysosomal enzymes, it does not internalize extracellular enzymes. At very high levels of overexpression, MPR-46 can mediate endocytosis (Watanabe *et al.*, 1990), however MPR-300-deficient cell lines such as M.H. 7777 neither bind nor internalise extracellular ligand. Lysosomal enzyme uptake by U937 cells, which express both types of MPR, is inhibited by antibodies specific for MPR-300, but not MPR-46. Internalization of anti-MPR-46 antibodies indicates that the failure of this receptor to mediate endocytosis is due not to its absence from the cell surface or an inability to be internalized, but rather to an inability of the cell surface receptor to bind ligand. The reason for this is unknown (Stein *et al.*, 1987d).

1.13.3 MPR-46 is involved in secretion of lysosomal enzymes

In addition to its role in transporting newly synthesized enzymes to lysosomes, MPR-46 is directly involved in their secretion (Chao, 1990). When cells expressing both receptors were transfected with MPR-46 cDNA, they secreted excessive amounts of M6P-containing proteins. Secretion was reduced by antibodies which blocked the MPR-46 binding site, indicating that these

proteins bound to MPR-46 prior to their secretion. Since co-transfection with MPR-300 negated the increase in secretion, these investigators concluded that MPR-46 was directly involved in secretion, and that enhanced secretion resulted from an imbalance of the two receptors in favour of MPR-46.

A model was proposed whereby the two receptors compete for binding of newly synthesized lysosomal enzymes in the Golgi: those bound by MPR-300 are transported to lysosomes, while MPR-46 transports its ligands either to the lysosomes or to a site from which they can exit the cell. As secreted enzymes are in precursor form, this site must be situated prior to the prelysosomal compartment and lysosomes, in which proteolytic processing of many lysosomal enzymes occurs. MPR-46 may release it's ligand directly at the cell surface, for the same reasons that prevent extracellular ligands from binding to cell surface MPR-46, or in early endosomes, where the free ligand would reach the cell surface as part of the fluid phase.

Table 1.1 Comparison of MPR-300 and MPR-46

Characteristic		MPR-300	MPR-46
Relative molecular weight	-polypeptide	275, 000	26, 500 - 28, 000
	- glycosylated	300, 000	46, 000 (varies with tissue source)
Structural comparison		Some sequence homology between extracytoplasmic domains. No homology between transmembrane and cytoplasmic domains.	
Extracytoplasmic domain		15 cysteine-rich repeat sequences with 16-38% sequence identity.	1 cysteine-rich domain; shares 14-28% homology with MPR-300 repeat sequences.
Oligomeric structure		Probably a monomer.	Monomer, dimer, or tetramer, depending onpH, and receptor or ligand concentration.
Ligand specificity		Both bind M6P phosphomonoesters, preferably linked α1,2 to an underlying mannose residue. •some affinity for phosphodiesters •has greater affinity for divalent ligands than does MPR-46 •binds IGF-II	•does not bind phospho-diesters
Number of M6P binding sites per monomer		2	1
Number of IGF-II binding sites per monomer		1	0
pH dependence of M6P binding	low pH	optimal at pH 6.0-6.3, decreases sharply below 6.0	
	neutral pH	slight decrease between 6.0-7.4	rapid decline between 6.0-7.4
Intracellular location		Co-expressed in most cells. About 90% expressed in intracellular membranes (Golgi, TGN, endosomes, coated vesicles). Approximately 10% on the plasma membrane.	
Role in lysosomal enzyme targeting		•Intracellular transport •Endocytosis of extra-cellular enzymes	•Intracellular transport •Secretion of enzymes
Role in signal transduction		IGF-II binding stimulates a G_i protein-mediated signal transduction pathway.	No known role in signal transduction.

1.14 Aims of this study

The aim of this study was to test the hypothesis proposed by Parish *et al.* (1990) that extracellular lysosomal enzymes, expressed on the cell surface of extravasating leukocytes by means of MPRs, contribute to their ability to degrade the subendothelial basement membrane. Under normal circumstances, a small percentage of lysosomal enzymes are secreted instead of being delivered to the lysosomes. This may occur directly, by means of MPR-46 (Chao, 1990), or by failure to bind either MPR in the *trans*-Golgi/TGN, such that the enzyme continues along the secretory pathway. Secreted enzymes may be recaptured and endocytosed by MPR-300 expressed at the cell surface. MPR-300 has a half-life of 3-5 minutes at the plasma membrane (Braulke *et al.*, 1987; Gonzalez-Noriega *et al.*, 1980), which is not affected by ligand binding (Jin *et al.*, 1989), thus bound lysosomal enzymes can remain at the cell surface for up to several minutes. This may provide sufficient time for any one enzyme to degrade surrounding basement membrane components. It is important to note that in this scenario, enzymes must be secreted prior to their delivery to the lysosomes, so that their M6P marker remains intact. Some inflammatory cells, such as macrophages, secrete bulk amounts of lysosomal enzymes by fusion of lysosomes with the plasma membrane (Henson, 1971; Movat *et al.*, 1964; Parish, 1969; Tew *et al.*, 1969; Weissman *et al.*, 1971). As these enzymes no longer carry the M6P marker, they cannot be displayed on the cell surface in the manner proposed here, and instead, must exert their effects in a soluble form.

The inhibition of T cell-mediated, passively induced EAE and adjuvant arthritis by CS and M6P, described in Section 1.8, provides indirect support for this hypothesis. This study sought to extend this work and provide more direct evidence that MPR-mediated expression of lysosomal enzymes on the cell surface of T lymphocytes could contribute to their degradation of the subendothelial basement membrane and subsequent entry into tissues. Two approaches were taken.

1. *Is MPR required for cell surface expression of degradative enzymes?*

Firstly, if MPR-300 is involved in the cell surface expression of degradative enzymes, then disrupting ligand binding should reduce the ability of extravasating cells to degrade the subendothelial basement membrane, by analogy with the proposed mechanism of action of M6P. The first part of this study aimed to specifically prevent lysosomal enzyme binding to cell surface MPR-300 using monoclonal antibodies specific for this receptor, and to evaluate

the effect on the degradative ability of T lymphocytes, both in terms of their ability to induce EAE *in vivo* and to degrade ECM laid down by cultured endothelial cells.

2. *Does T lymphocyte activation affect MPR expression?*

Adoptive transfer of EAE is effected by activated, MBP-specific T cells. In recent years, it has become apparent that the CNS is randomly surveyed by T cells, and can be entered by activated T cell blasts, regardless of their antigen specificity. Wekerle *et al.* (1986) obtained preliminary evidence that activated T cells specific for non-CNS antigens could enter the CNS, and proposed that emigration of lymphocytes into the CNS depends only on the activation state of the cell. Subsequent experiments have confirmed that activated, but not resting, T cells enter the CNS (Hickey *et al.*, 1991; Ludowyk *et al.*, 1992). *In vitro* studies have also shown that activated T cells are more adhesive for endothelium, and that secretion of matrix-degrading proteases and heparanases is enhanced in comparison with resting cells (Ebnet *et al.*, 1991; Naparstek *et al.*, 1984; Savion *et al.*, 1984; Simon *et al.*, 1991), providing some insight into the mechanisms behind their invasive behaviour. Parish and coworkers have proposed that T cell activation also increases their invasive potential by increasing cell surface expression of lysosomal enzymes. If lysosomal enzymes are expressed on the cell surface by means of MPR-300, activation could bring about an increase in their cell surface expression by either or both of the following.

a) Increasing the expression of MPR-300 at the cell surface, thus increasing the number of lysosomal enzyme binding sites available.

b) Increasing the availability of ligand by an increased secretion of lysosomal enzymes, assuming that cell surface MPR-300 on the resting cell is not saturated with ligand. An increase in lysosomal enzyme secretion could be accomplished by several mechanisms:

i) Increased synthesis of a specific lysosomal enzyme

Expression of sufficiently large amounts of any enzyme saturates the available M6P binding sites in the *trans*-Golgi and TGN, and excess enzyme is secreted via the secretory pathway. Other lysosomal enzymes are also secreted, as they compete for the same binding sites. This is illustrated in hormone-dependent breast cancer cells, in which estrogen stimulates overexpression of cathepsin D and down-regulates MPR-300.

The depleted levels of intracellular MPR-300 are saturated by their estrogen-induced ligand, and consequently the secretion of both cathepsin D and other lysosomal enzymes, produced in lesser amounts, is increased (Capony *et al.*, 1990; Mathieu *et al.*, 1991).

Secretion of lysosomal enzymes can also arise from incorrect synthesis of the M6P recognition marker. This is seen in I cell disease, where most lysosomal enzymes are affected (Hickman and Neufeld, 1972; Neufeld and Cantz, 1971), and in transformed murine fibroblasts, where cathepsin L has an unusually low affinity for MPR, and is selectively secreted (Dong *et al.*, 1989; Dong and Sahagian, 1990; Sahagian and Gottesman, 1982). As the secreted enzymes in these examples bind inefficiently to MPR, this means of increasing the concentration of extracellular lysosomal enzymes is inappropriate in the context of our model, as they bind equally poorly to cell surface MPR-300 as to the intracellular receptor.

ii) Changes in the distribution of either or both of MPR-300 and MPR-46

MPR-300 and MPR-46 are expressed simultaneously in most cells, have a similar intracellular distribution (Bleekmolen *et al.*, 1988; Gabel *et al.*, 1983), and compete for binding of newly synthesized lysosomal enzymes in the *trans*-Golgi and TGN. While both are involved in intracellular transport to lysosomes, MPR-46 has also been reported to transport enzymes directly to the cell surface (Chao, 1990). Secretion of lysosomal enzymes could thus be enhanced by increasing the proportion of MPR-46 in the Golgi region. Fewer lysosomal enzymes would then be directed to lysosomes by MPR-300, and more to the cell surface by MPR-46.

To summarize, T cell activation could increase cell surface binding of lysosomal enzymes by increasing the expression of MPR-300 at the cell surface, and/or the expression of MPR-46 in the Golgi. This could be achieved by a redistribution of the existing MPR pool, with or without an increase in receptor synthesis. The second part of this study aimed to use mAbs specific for MPR-300 and MPR-46 to compare the expression of the two receptors in resting and activated T lymphocytes.

CHAPTER 2

DEVELOPMENT OF AN ASSAY TO DETECT MPR-300 EXPRESSION ON THE CELL SURFACE

2.1 INTRODUCTION

Parish *et al.* (1990) have proposed that lysosomal enzymes, displayed on the surface of extravasating T cells by MPRs, participate in the degradation of the subendothelial basement membrane. The aim of the first part of this study was to examine the consequences of preventing lysosomal enzyme binding to the T cell surface, using monoclonal antibodies (mAbs) specific for rat MPR-300. This chapter describes a simple assay for detecting MPR-300 on the surface of intact cells, based on the binding and endocytosis of a fluorescent, M6P-rich ligand. This assay was developed to provide a means of (a) identifying a rat cell line with sufficiently high expression of MPR-300 on the cell surface for screening hybridoma supernants for binding to the extracellular domain of the receptor; and (b) screening antibodies for their ability to interfere with ligand binding to cell surface MPR-300.

In previous studies, cell surface and intracellular MPR in intact cells and tissue sections have been detected by immunofluorescence, immuno-electronmicroscopy and immunocytochemistry at the light microscopic level, using antisera specific for MPR-300 and MPR-46 (Brown *et al.*, 1984; Brown and Farquhar, 1984b; Geuze *et al.*, 1984a; Geuze *et al.*, 1988; Griffiths *et al.*, 1988; Matovcik *et al.*, 1990; Prydz *et al.*, 1990; Willingham *et al.*, 1983). While antibody binding is a sensitive assay for the presence of receptors, and is used in later chapters to detect cell surface MPR-300 on leukocytes, it could not be used to screen mAbs for their ability to interfere with ligand binding. For this purpose, a functional assay dependent on ligand binding was required.

Other studies concerned with ligand characterization, and the kinetics of ligand binding and MPR-300 internalization, have utilized assays involving either ligand binding to membranes or whole cells at 4°C, or uptake by cells at 37°C. Ligands include lysosomal enzymes, such as mammalian β-glucuronidase (Fischer *et al.*, 1980a; Kaplan *et al.*, 1977a; Kaplan *et al.*, 1977c; Kaplan *et al.*, 1978; O'Brien *et al.*, 1989), β-galactosidase (Distler *et al.*, 1979; Sahagian *et al.*, 1979), β-hexosaminidase B (Fischer *et al.*, 1980b) and L-iduronidase (Rome *et al.*, 1979; Sando, 1978; Sando and Neufeld, 1977), and α-mannosidase from the slime mold *Dictyostelium discoideum* (Shepherd *et al.*, 1984). These lysosomal enzymes were purified from cellular secretions rather than whole cells, as an intact M6P marker is required for binding to MPR. Alternative ligands include phosphorylated oligosaccharides, such as high mannose-type oligosaccharides derived from lysosomal enzymes (Natowicz *et al.*, 1983), and pentamannose

phosphate (PMP) conjugated to BSA (Braulke *et al.*, 1987). Detection of these ligands has relied on radioactive labelling, or assaying for cell-associated enzymatic activity.

While these methods fulfilled the requirement for an assay dependent on ligand binding, detection of the bound or endocytosed ligand were technically laborious. For use in antibody screening, a rapid and simple assay was required. This chapter describes an assay based on those described above, in which binding and uptake of a highly fluorescent, M6P-rich polysaccharide was measured by flow cytometry.

The MPR ligand was isolated from the exopolysaccharide secreted by the yeast *Pichia holstii*. Acid hydrolysis of yeast exopolysaccharides yields a phosphomannan monoester core polysaccharide (PPME), containing many exposed M6P residues, and smaller pentamannose phosphate (PMP) fragments (Fischer *et al.*, 1980c). Phosphomannans and their partial hydrolysis products competitively inhibit lysosomal enzyme uptake by fibroblasts (Fischer *et al.*, 1980c; Kaplan *et al.*, 1977a; Kaplan *et al.*, 1978), and biosynthetically-labelled PPME and PMP bind saturably to the cell surface of fibroblasts. The multivalent PPME has a high affinity for cell surface MPR, is a potent inhibitor of lysosomal enzyme uptake, and is endocytosed efficiently (Fischer *et al.*, 1980c). This chapter describes the binding and uptake of fluorescein-labelled PPME by the U937 cell line.

2.2. EXPERIMENTAL PROCEDURES

2.2.1 Materials

Rabbit antiserum raised against bovine MPR-300, and cross-reactive with human MPR-300, was a generous gift from Dr G. Gary Sahagian (Tufts University, Boston, MA). Fluorescein-labelled PPME (fl-PPME), purified from *Pishia holstii*, and fluorescein-labelled mannan (fl-mannan) were a gift from Dr Susan Weston (Manchester University, Manchester, UK). Phosphosugars and D-mannose were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2.2 Cell culture

The cell lines U937, Clone 9, BRL-3A and McA RH7777 were obtained from the American Type Culture Collection (Rockville, MD), and the P388D1 cell line from Dr Carolyn Geczy (Heart Research Institute, Sydney, Australia). U937, $P388D_1$ and Clone 9 cells were maintained at 37°C (5% CO_2) in RPMI 1640

medium (Gibco, Gaithersburg, MD), supplemented with 10% foetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia), 2 mg/ml sodium bicarbonate, 0.2 mM glutamine, 30 µg/ml penicillin, 50 µg/ml streptamycin sulfate and 50 µg/ml neomycin sulfate. BRL-3A were cultured in RPMI 1640 medium as above, supplemented with 5% FCS. McA RH7777 cells were maintained at 37°C (10% CO_2) in Dulbecco's modified Eagle's medium (DMEM; #430-1600, Gibco, Gaithersburg, MD) supplemented with 20% FCS, 3.7 mg/ml sodium bicarbonate, 4 mM glutamine, 3.5 mg/ml glucose, 120 µg/ml penicillin, 200 µg/ml streptomycin sulfate and 200 µg/ml neomycin sulfate.

U937 cells were subcultured by inoculation into fresh medium at a density between 5×10^4 and 1×10^5 cells/ml, and were grown to a maximum density of 1×10^6 cells/ml. Adherent cells (P388D1, Clone 9, BRL-3A and McA-RH7777) were released for subculturing with 0.25% trypsin (Cytosystems, Sydney, Australia) and 0.1% EDTA in PBS when monolayers were confluent. The subcultivation ratio was between 1:6 and 1:30 for Clone 9 cells, 1:4 for BRL-3A and $P388D_1$ cells, and between 1:2 and 1:6 for McA RH7777 cells.

2.2.3 Immunofluoresent flow cytometry

U937 cells were treated with rabbit antiserum, washed and incubated with a fluorescein-Protein A conjugate (Pharmacia, Uppsala, Sweden) at 100 µg/ml. Incubations were in 96-well V-bottomed plastic plates (Nunc, Roskilde, Denmark) using 1×10^5 cells/ml in 40 µl volumes. All incubations were for 30 min on ice, and cells were washed three times with 10% FCS/RPMI between the two incubations. The final incubation was followed by two washes with 10% FCS/RPMI and one with PBS. Cells were fixed with 1% paraformaldehyde in PBS (pH 7.4) and analysed for fluorescence by flow cytometry (Section 2.2.5).

2.2.4 Assays for binding and uptake of fl-PPME

2.2.4.1 Binding and uptake of fl-PPME by cells in suspension

Where appropriate, adherent cells were released using 0.1% EDTA in PBS. Cells were resuspended in 10% FCS/RPMI medium and incubated with fl-PPME (up to 100 µg/ml) or fl-mannan, with or without inhibitors, for 60 min at 4°C (binding assays) or 3 h at 37°C (uptake assays), except where otherwise stated. Incubations were in 96 well V-bottomed plastic microtitre plates, using 1×10^5 cells in 40 µl volumes. Cells were washed twice with 10% FCS/RPMI

and once with PBS, then fixed with 1% paraformaldehyde in PBS and analysed for fluorescence by flow cytometry. Background autofluorescence of cells incubated with medium alone was subtracted from all samples.

2.2.4.2 Binding and uptake of fl-PPME by adherent cells

Adherent cells were released from flasks using 0.25 % trypsin and 0.1% EDTA in PBS, and resuspended in the appropriate medium at the same density used for subculturing. Monolayers were prepared in 24-well tissue culture plates (Linbro, ICN Biomedicals, Costa Mesa, CA) by adding 2 ml of the cell suspension per well, and incubating at 37°C until confluent. Monolayers were washed twice with 10% FCS/RPMI and incubated with 200 µl of fl-PPME or fl-mannan, with or without 2.5 mM M6P, for 3 h at 37°C. Cells were washed twice with 10% FCS/RPMI and released from the wells with 0.25% trypsin. The cells were washed again with RPMI medium, then with PBS and fixed in 1% paraformaldehyde in PBS. Cells were analysed for fluorescence by flow cytometry.

2.2.5 Flow cytometry

Cells were analysed for fluorescence using a FACScan (Becton Dickinson, Mountain View, CA) and Lysys II software. Cell populations were gated on the basis of forward and side scatter to exclude dead and aggregated cells, and results expressed as median fluorescence intensity units (FIU).

2.2.6 Confocal microscopy

The intracellular location of fl-PPME taken up by U937 cells was analysed by confocal microscopy, using an MRC-500 Confocal Imaging System (Biorad, Abingdon, Oxfordshire, UK) controlled by a dedicated computer system and equipped with a 25 mW multiline argon ion laser and Nikon Optiphot fluoresence microscope (Nikon, Tokyo, Japan).

2.3 RESULTS

2.3.1 Cell surface expression of MPR-300 on U937 cells

The human monocyte cell line U937 was used to develop an assay for detecting cell surface expression of MPR, as it has a relatively high content of both MPR-300 and MPR-46, with about 12% of each receptor found at the cell surface (Bleekmolen *et al.*, 1988). Initially, a rabbit antiserum specific for bovine MPR-300, and cross reactive with the human receptor, was used to confirm the presence of MPR-300 on the cell surface of U937 cells. Antibody binding was detected by immunofluorescent flow cytometry and is illustrated in Fig. 2.1. Antibody binding was maximal at 1/100 - 1/200 dilution and was substantially greater than binding of a control rabbit antiserum, consistent with cell surface expression of MPR-300 on these cells.

PPME binding was examined by incubating U937 cells with fl-PPME for 60 min at 4°C. The dependence of binding on ligand concentration is shown in Fig. 2.2. Non-specific binding was determined as binding of fl-PPME in the presence of 5 mM M6P. The M6P-specific component of binding appeared saturable, consistent with binding to a cell surface receptor (Warlow, 1989).

The extent of fl-PPME binding varied considerably between experiments, with cell-associated fluorescence varying by as much as 100% (data not shown). The M6P-inhibitable component of the fluorescence was quantitatively low (6-11 FIU at 50 μg/ml PPME), and was not increased by preincubating the cells with M6P in order to remove endogenous lysosomal enzymes (data not shown). Thus, endocytosis of PPME was examined so as to increase the sensitivity of the assay.

2.3.2 Uptake of fl-PPME by U937 cells

A more sensitive variant of the PPME binding assay was developed by exploiting the ability of cell surface MPR to bind and internalize ligand. Fig. 2.3 illustrates uptake of fluorescent PPME as a function of ligand concentration. The degree of uptake varied between experiments, as was the case for fl-PPME binding, but results were qualitatively similar each time. Non-specific uptake was determined by including 5 mM M6P in the assay. As fl-PPME uptake in the presence of M6P was very low over the entire concentration range, it is likely to be mediated entirely by a receptor for M6P. However, since PPME contains a high proportion of mannose residues, with a mannose: phosphate

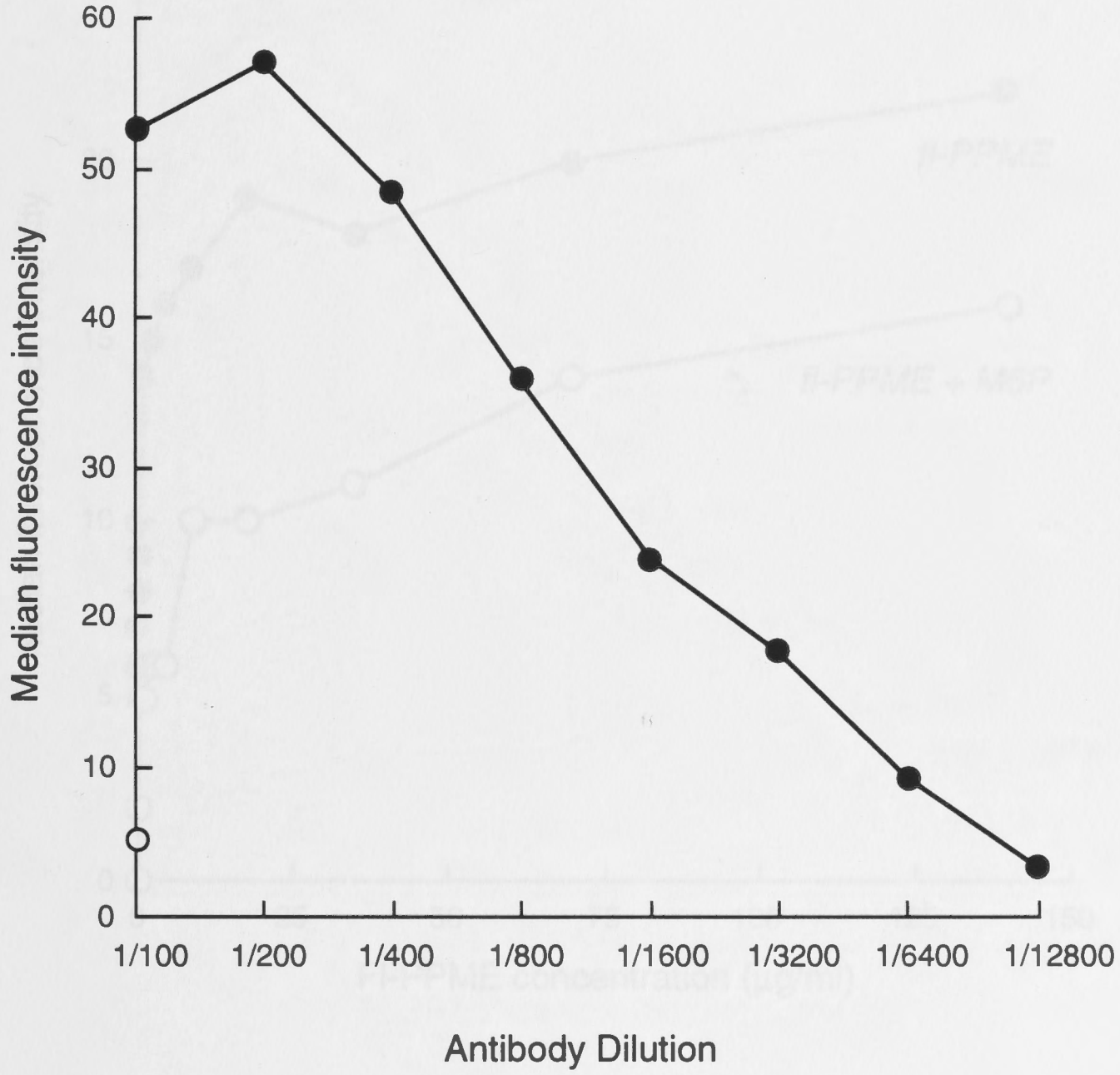


Fig. 2.1

Binding of a polyclonal rabbit anti-MPR-300 antiserum to U937 cells. Cells (1×10^5) were incubated with anti-MPR-300 antiserum (●) for 30 min at 4°C, and antibody binding measured by immunofluorescent flow cytometry. All values are corrected for background autofluorescence. Fluorescence is compared with that due to binding of a rabbit antiserum (1/100 dilution) specific for chicken histidine-rich glycoprotein (O).

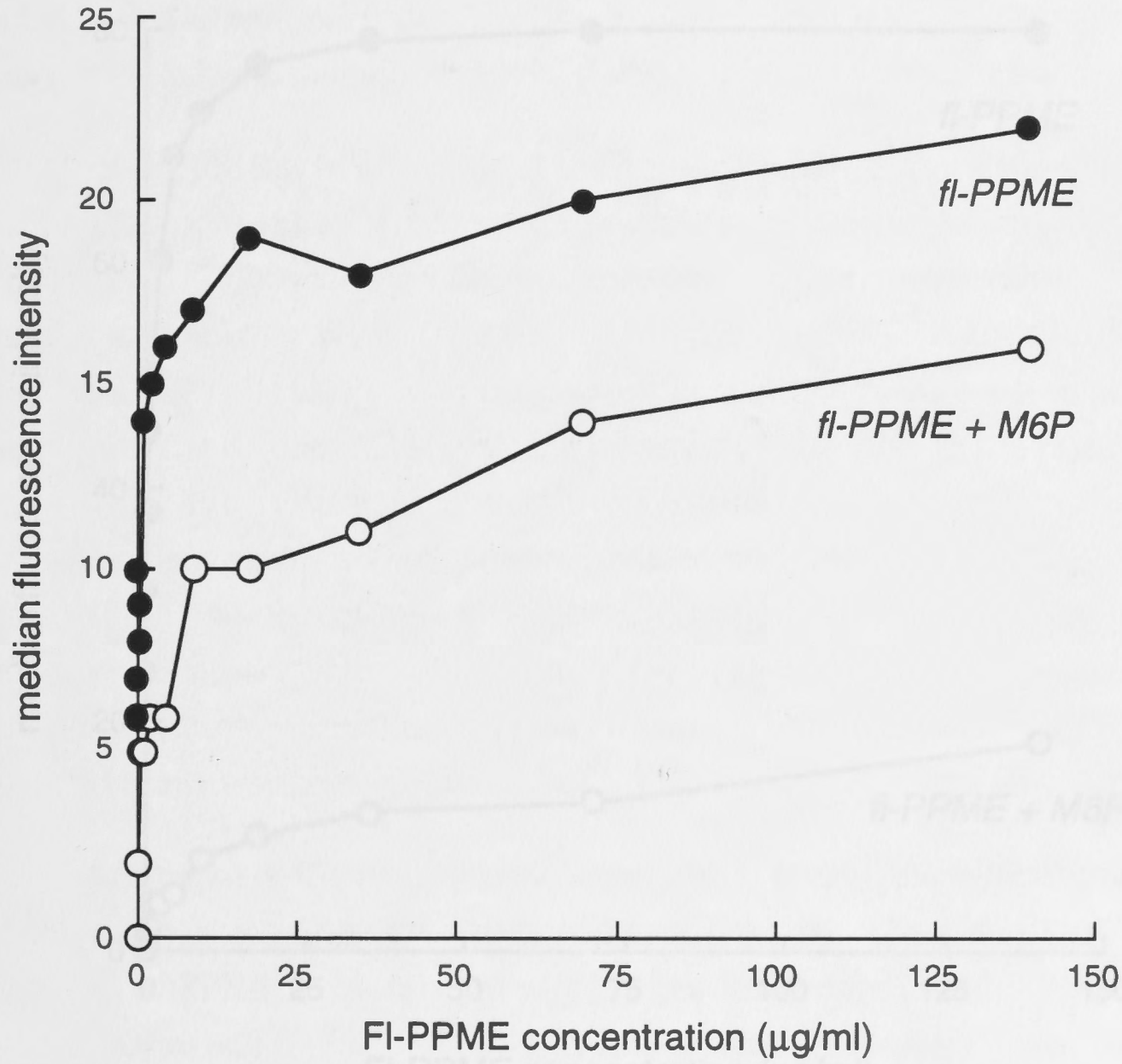


Fig. 2.2

Binding of fl-PPME to U937 cells (1×10^5) incubated with increasing concentrations of ligand for 60 min at 4°C. Cell-associated fluorescence was determined by flow cytometry. Non-specific binding was determined by adding 5 mM M6P to companion assays. All values are corrected for background autofluorescence. Single data points are shown, however similar results were obtained in 9 separate experiments.

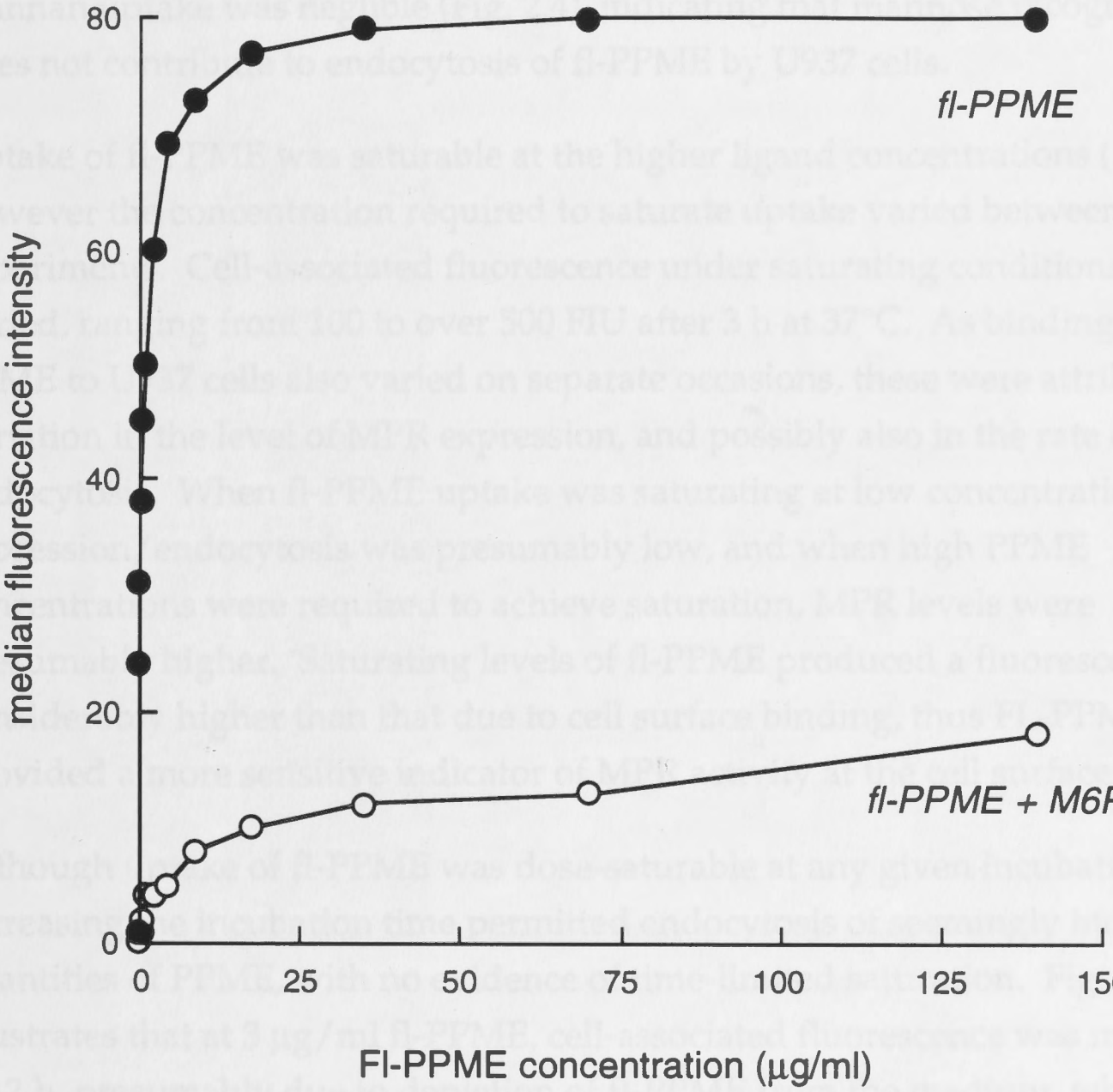


Fig. 2.3

Uptake of fl-PPME by U937 cells (1×10^5) incubated with increasing concentrations of ligand for 3 h at 37°C. Cell-associated fluorescence was determined by flow cytometry. Non-specific binding was determined by adding 5 mM M6P to companion assays. All values are corrected for background autofluorescence. Single data points are shown, however similar results were obtained in 10 separate experiments.

ratio of 20:1 (Weston, 1991)), mannose-specific uptake was also determined. Mannose itself is a poor inhibitor of mannose-specific uptake (Lennartz *et al.*, 1987; Stephenson and Shepherd, 1987), so uptake of fl-PPME and fl-mannan (a yeast polysaccharide consisting only on mannose residues) were compared. Fl-mannan uptake was neglible (Fig. 2.4), indicating that mannose recognition does not contribute to endocytosis of fl-PPME by U937 cells.

Uptake of fl-PPME was saturable at the higher ligand concentrations (Fig. 2.3), however the concentration required to saturate uptake varied between experiments. Cell-associated fluorescence under saturating conditions also varied, ranging from 100 to over 500 FIU after 3 h at 37°C. As binding of fl-PPME to U937 cells also varied on separate occasions, these were attributed to variation in the level of MPR expression, and possibly also in the rate of endocytosis. When fl-PPME uptake was saturating at low concentrations, MPR expression/endocytosis was presumably low, and when high PPME concentrations were required to achieve saturation, MPR levels were presumably higher. Saturating levels of fl-PPME produced a fluorescent signal considerably higher than that due to cell surface binding, thus FL-PPME uptake provided a more sensitive indicator of MPR activity at the cell surface.

Although uptake of fl-PPME was dose-saturable at any given incubation time, increasing the incubation time permitted endocytosis of seemingly indefinite quantities of PPME, with no evidence of time-limited saturation. Fig. 2.5 illustrates that at 3 µg/ml fl-PPME, cell-associated fluorescence was maximal by 2 h, presumably due to depletion of fl-PPME from the medium, while at 50 µg/ml, PPME was endocytosed almost linearly over a 5 h period. Despite the availability of 20 times as much fl-PPME in the latter assay, cell-associated fluorescence after 5 h was only 5 fold higher than in the cells incubated with 3 µg/ml PPME, suggesting that they could continue to endocytose PPME for much longer. This is consistent with the continuous recycling of MPRs between sites of ligand binding and release (Section 1.9).

Confocal micrographs of cells incubated with PPME at 37°C showed that the internalized ligand is compartmentalized (Fig. 2.6), consistent with release of fl-PPME in the prelysosomal compartment, and subsequent delivery to lysosomes (Section 1.12.2). Fl-PPME uptake was also inhibited in the presence of chloroquine, a lysosomotropic amine which reduces cell surface expression of MPR-300 (Braulke *et al.*, 1987; Gonzalez-Noriega *et al.*, 1980), and also raises the pH of acidic organelles (Shoji and Poole, 1978), preventing ligand-MPR dissociation within the prelysosomal compartment (Brown *et al.*, 1984). After a

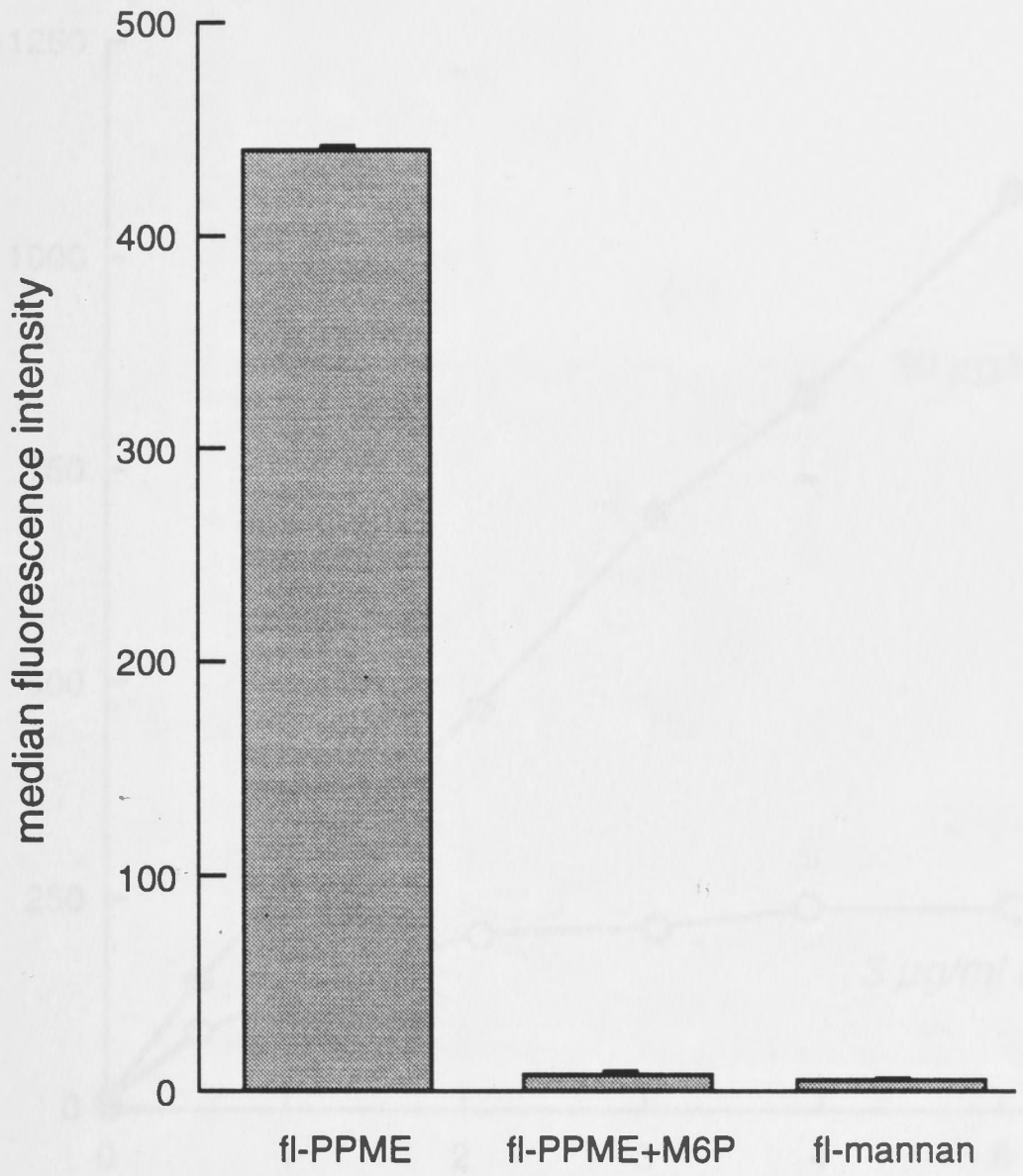


Fig. 2.4

Comparison of uptake of fl-PPME and fl-mannan by U937 cells. Cells (1×10^5) were incubated with fl-PPME or fl-mannan (50 µg/ml) for 3 h at 37°C. Non-specific uptake of fl-PPME was determined by including 5 mM M6P in a companion assay. Cell-associated fluorescence was determined by flow cytometry. All values are corrected for background autofluorescence. Values represent mean ± SEM (n=3).

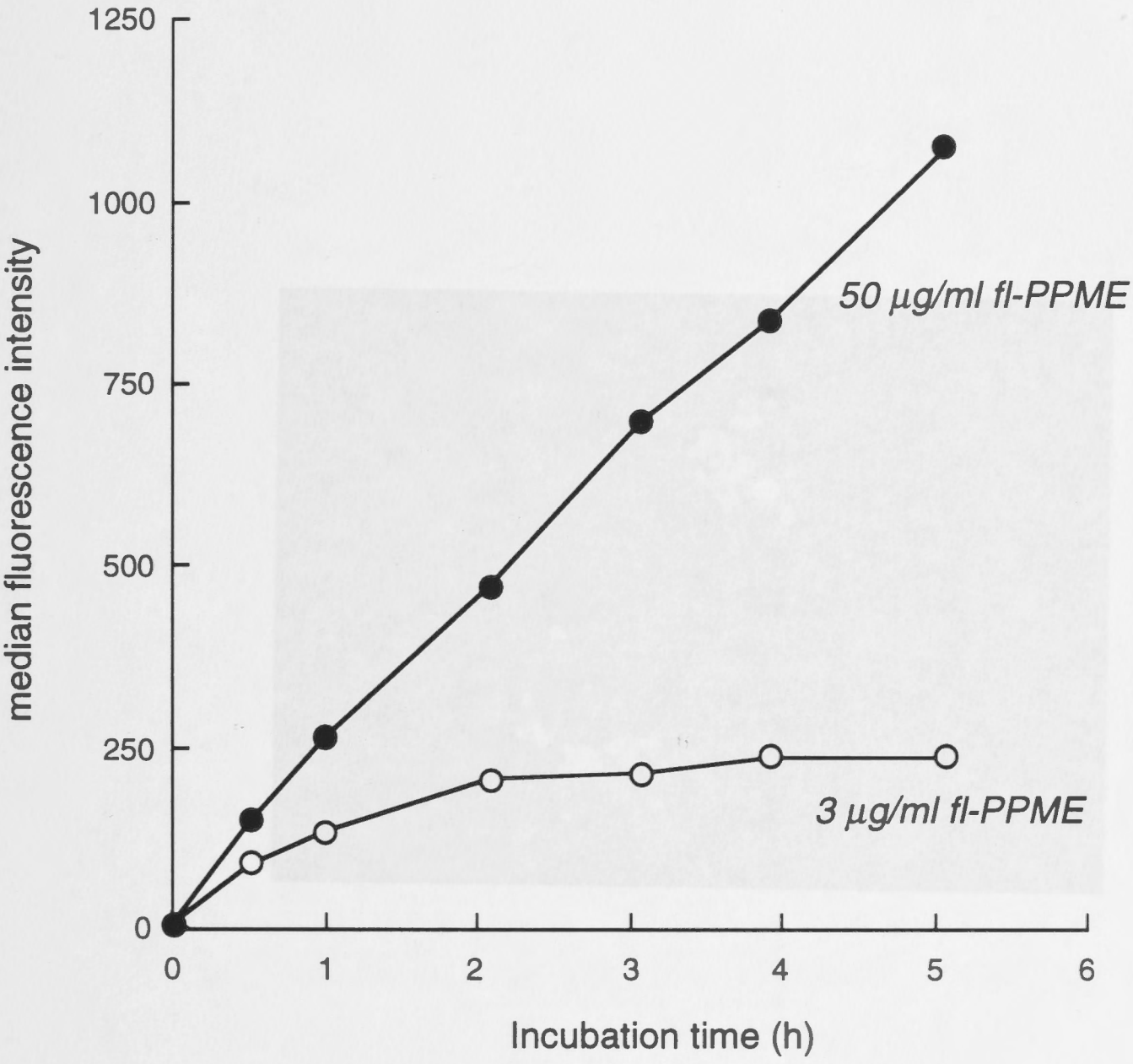


Fig. 2.5

Time course of fl-PPME uptake by U937 cells (1×10^5) incubated at 37°C with fl-PPME at concentrations of 3 µg/ml and 50 µg/ml. Cell-associated fluorescence was determined by flow cytometry. All values are corrected for background autofluorescence. Single data points are shown, but similar results were obtained in two separate experiments.

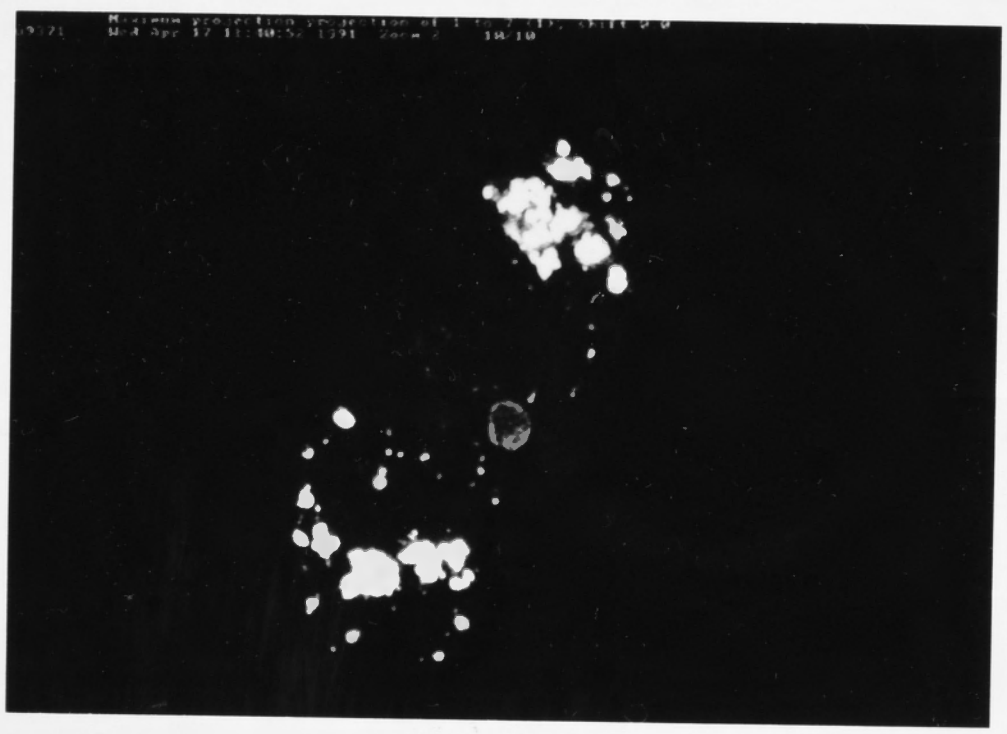


Fig. 2.6

Intracellular destination of endocytosed fl-PPME. U937 cells (1×10^5) were incubated with fl-PPME (50 µg/ml) for 6 h at 37°C. Washed cells were fixed with 1% paraformaldehyde and examined by confocal fluorescence microscopy.

5 h incubation in the presence of 25 mM chloroquine, cell-associated fluorescence was only 15 % of the control level (Fig. 2.7). This is consistent with the effect of chloroquine on MPRs; by preventing ligand release in the acidified prelysosomal/endosomal compartment, chloroquine depletes the cell of unoccupied receptors, thus preventing endocytosis of exogenous ligand (Gonzalez-Noriega *et al.*, 1980).

The sugar specificity of fl-PPME uptake was tested using several phosphosugars as inhibitors (Fig. 2.8). Fl-PPME uptake by U937 cells was strongly inhibited by M6P and fructose 1-phosphate (F1P). Fructose 6-phosphate (F6P) and glucose 6-phosphate (G6P) weakly inhibited PPME uptake, while the effects of galactose 6-phosphate (Gal6P) and mannose 1-phosphate (M1P; data not shown) were little better than mannose.

2.3.4 The contribution of MPR-300 to fl-PPME uptake

Although both MPR-300 and MPR-46 are found on the plasma membrane of U937 cells, only MPR-300 binds extracellular M6P-bearing ligands (Stein *et al.*, 1987d). It is probable then that fl-PPME uptake is mediated by MPR-300 alone. To confirm this, two cell lines which do not express MPR-300 were incubated with fl-PPME at 4°C and 37°C. M6P-specific binding and uptake of fl-PPME by the murine P388D1 macrophage cell line (Gabel *et al.*, 1983; Hoflack and Kornfeld, 1985a) was very low, and negligible compared to that observed for U937 cells (Fig. 2.9 (a, b)). The McA RH7777 cell line is derived from the rat M.H. 7777 hepatoma line, which has also been reported not to express MPR-300 (Mainferme *et al.*, 1985). McA RH7777 cells also showed no M6P-specific binding or uptake of PPME (data not shown). This suggests that fl-PPME uptake is specific for MPR-300.

2.3.4 Detection of cell surface MPR-300 on rat cell lines

A rat cell line was required for screening hybridoma supernatants for binding to cell surface MPR. Two rat hepatocyte cell lines, Clone 9 and BRL-3A, were obtained and tested for M6P-specific uptake of fl-PPME. Adherent cells of both types endocytosed fl-PPME in a M6P-dependent manner, however M6P-specific uptake by Clone 9 cells was 35-fold higher than by BRL-3A cells (Fig. 2.10). As non-adherent cells were required for the antibody screening assay, Clone 9 cells were detached using EDTA and examined for uptake of fl-PPME. The detached cells bound and endocytosed fl-PPME in an M6P-inhibitable manner, although less efficiently than did the adherent cells (Fig. 2.11). The

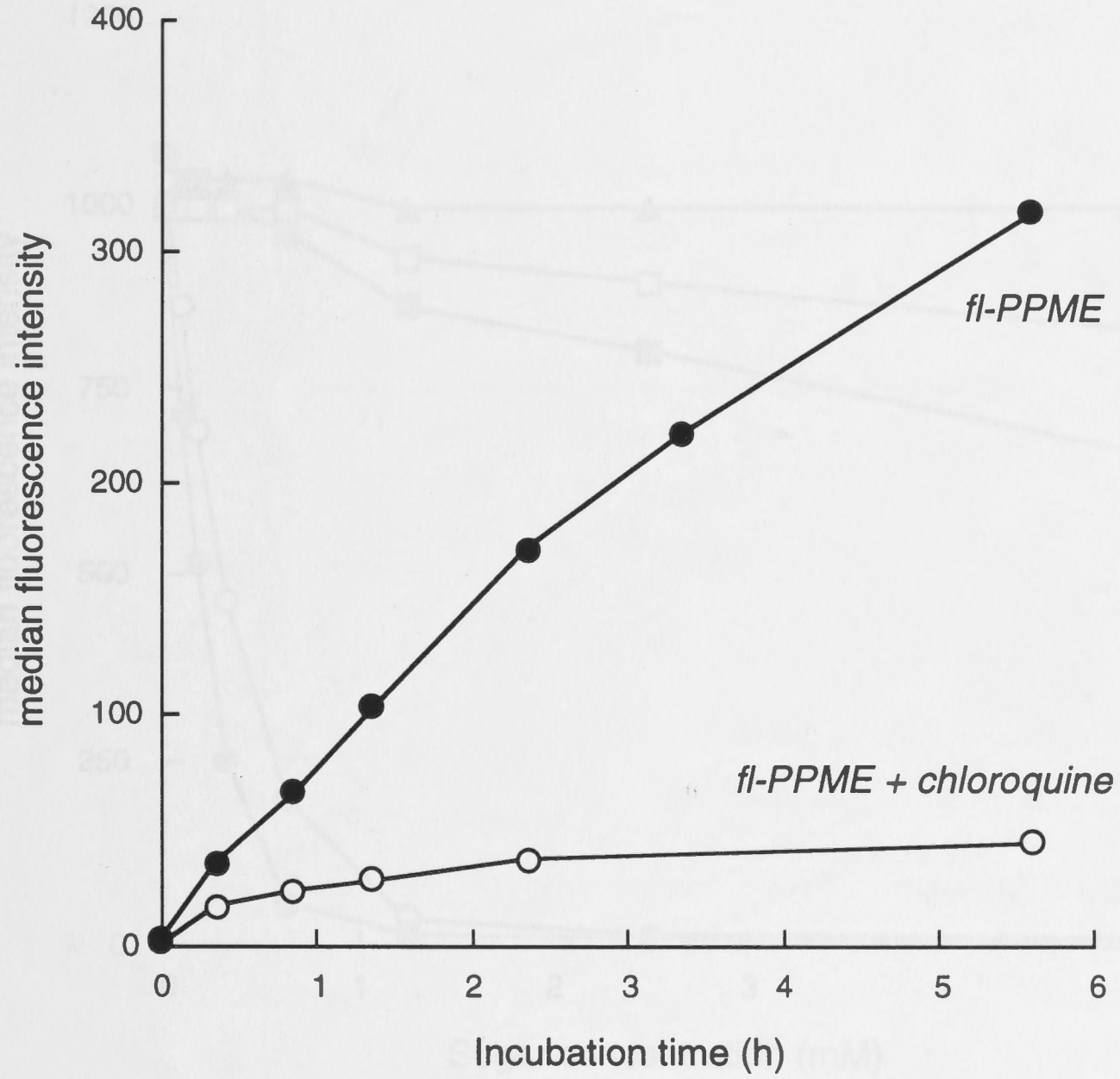


Fig. 2.7

Effect of chloroquine on uptake of fl-PPME by U937 cells. Cells (1×10^5) were incubated with 50 µg/ml fl-PPME for varying times at 37°C in the presence and absence of 25 mM chloroquine. Cell-associated fluorescence was determined by flow cytometry. All values are corrected for background autofluorescence. Single data points are shown.

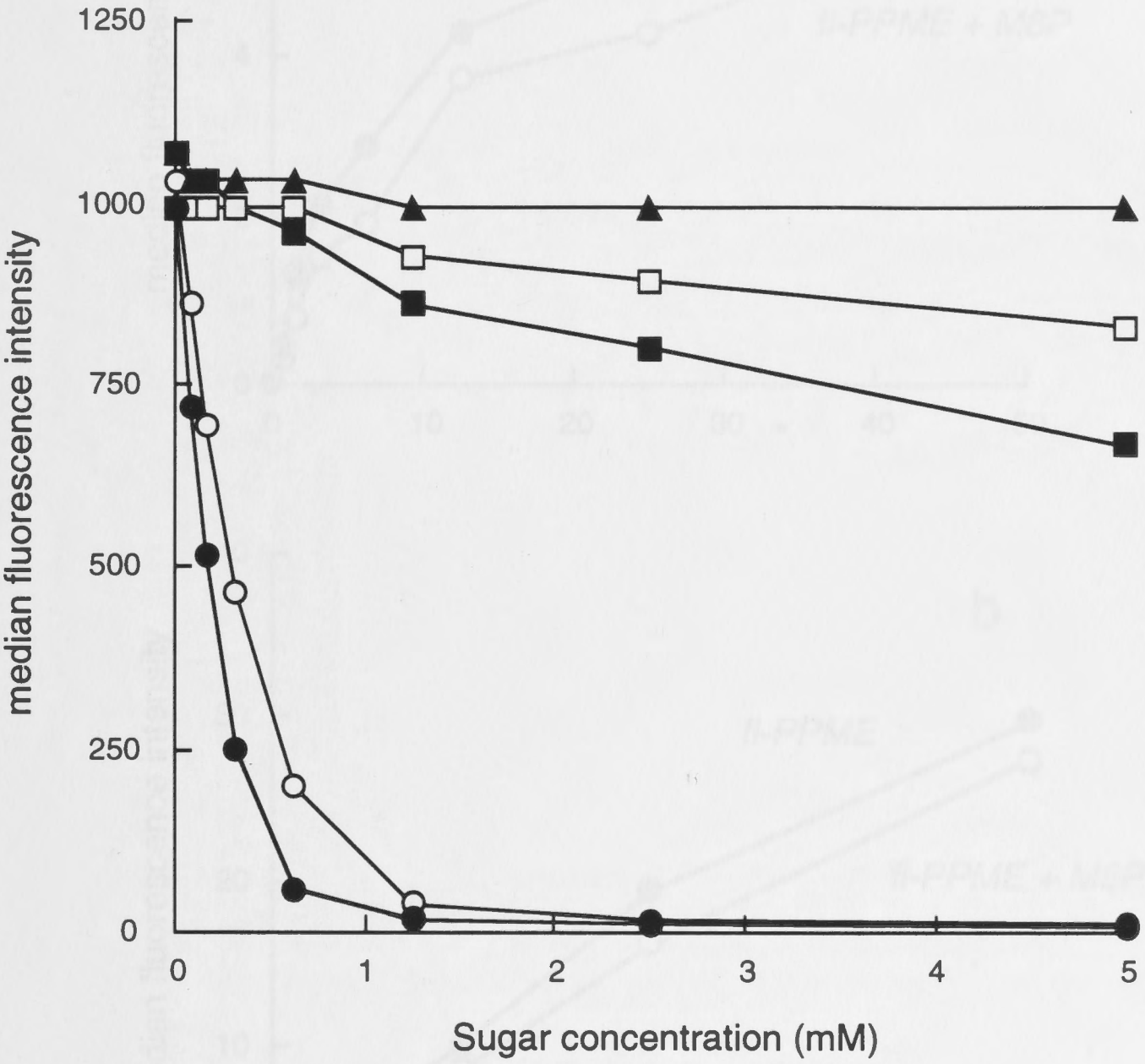


Fig. 2.8

Inhibition of fl-PPME uptake by a range of phosphosugars and mannose. U937 cells (1×10^5) were incubated with fl-PPME (25 µg/ml) for 4 h at 37°C in the presence of varying concentrations of mannose 6-phosphate (●), fructose 1-phosphate (○), fructose 6-phosphate (■), glucose 6-phosphate (□) and mannose (▲). Cell-associated fluorescence was determined by flow cytometry. All values are corrected for background autofluorescence. Single data points are shown, however similar results were obtained in two separate experiments.

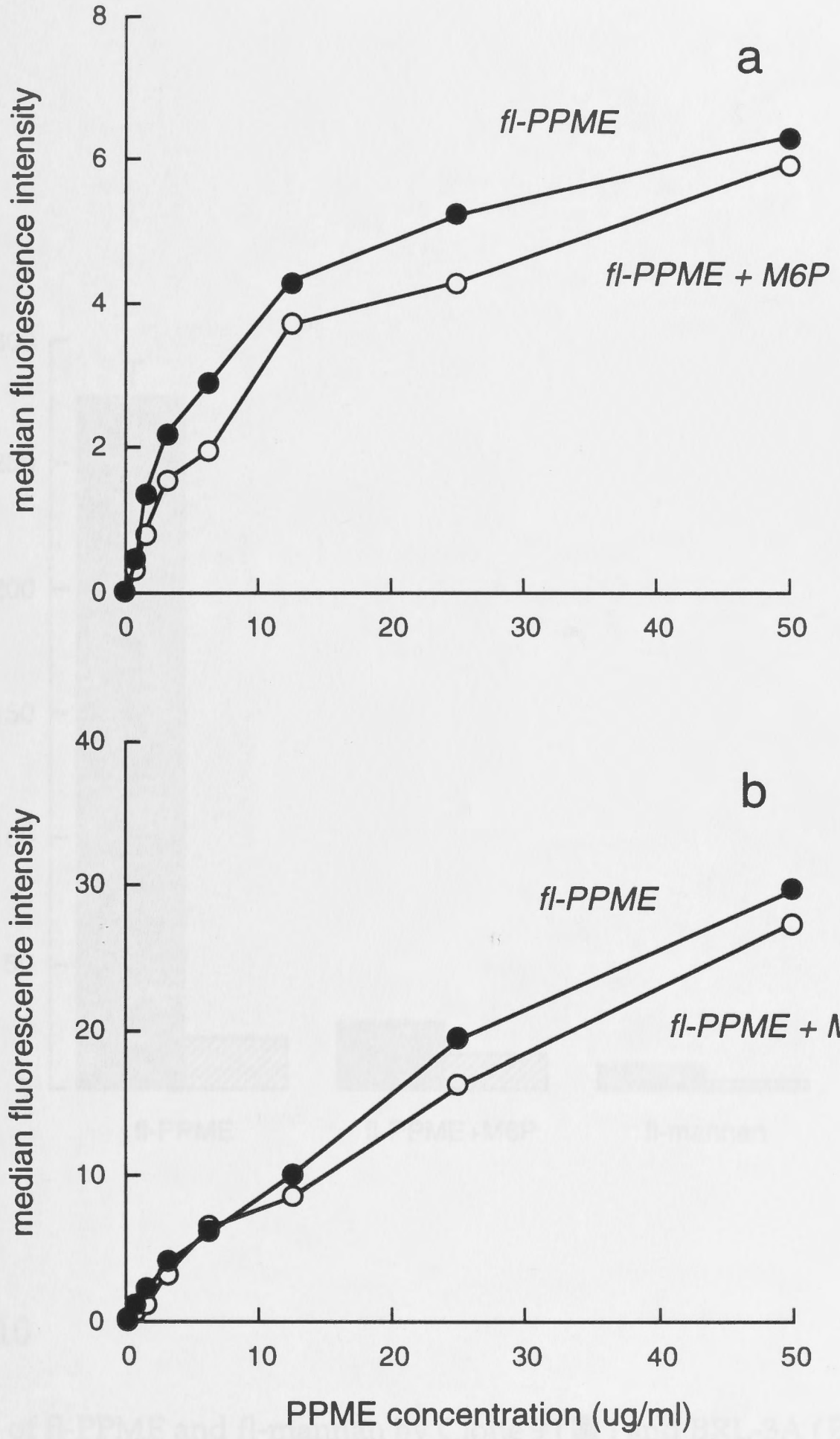


Fig. 2.9

Binding (a) and uptake (b) of fl-PPME by P388D1 murine macrophages. Adherent cells were detached with 0.1% EDTA and incubated with fl-PPME (50 µg/ml) for 1 h at 4°C (a), or 3 h at 37°C (b). Non-specific binding/uptake was determined by including 5 mM M6P in companion assays. Cell-associated fluorescence was determined by flow cytometry. All values are corrected for background autofluorescence. Single data points are shown, but similar results were obtained in two separate experiments.

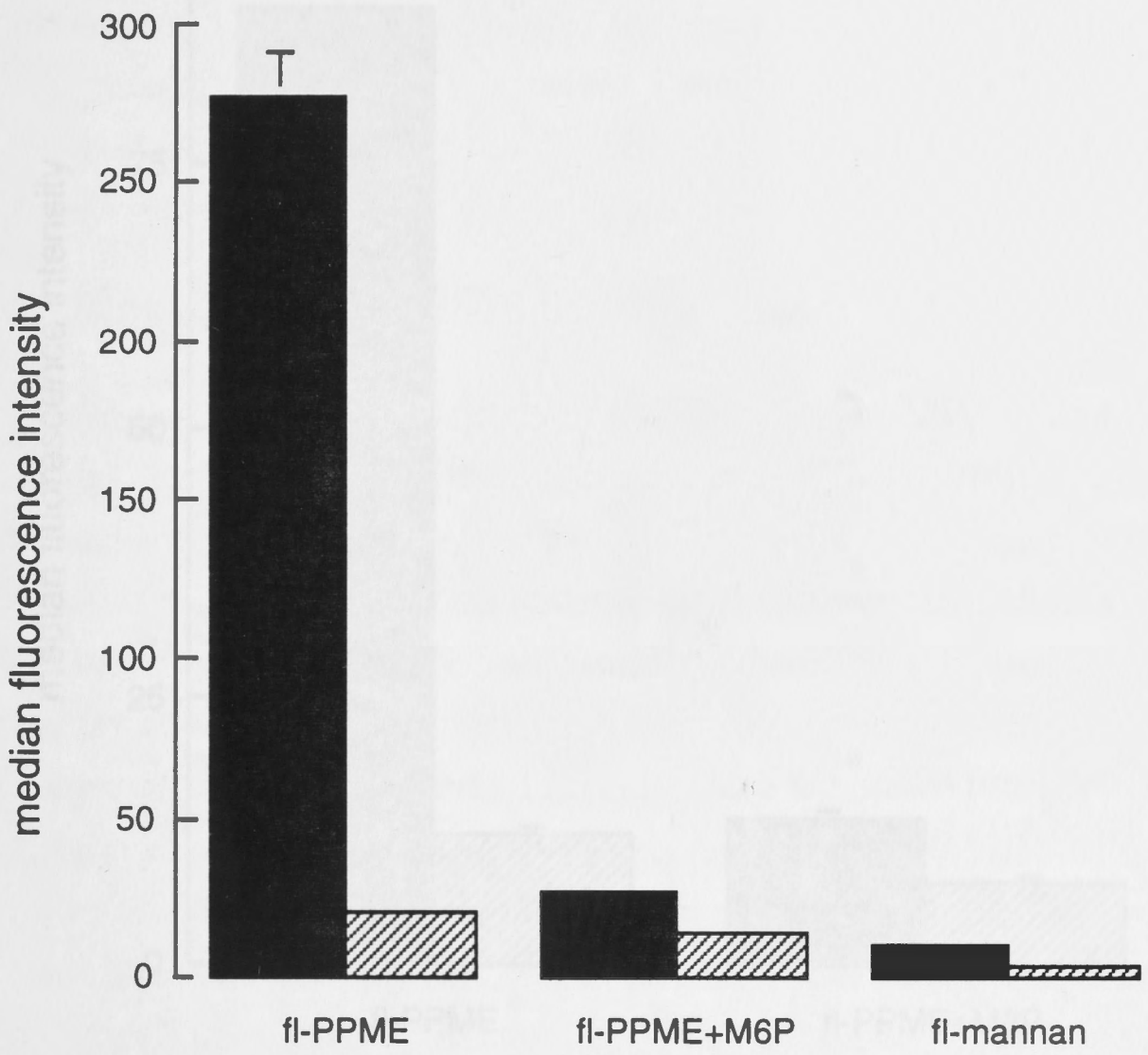


Fig. 2.10

Uptake of fl-PPME and fl-mannan by Clone 9 (■) and BRL-3A (▨) rat hepatocytes. Adherent cells grown in 24-well plates were incubated with fl-PPME or fl-mannan (25 µg/ml) for 3 h at 37°C. Non-specific uptake of fl-PPME was determined by including 2.5 mM M6P in a companion assay. Adherent cells were released with trypsin and cell-associated fluorescence was determined by flow cytometry. All values are corrected for background autofluorescence. Values represent mean ± SEM (n=3).

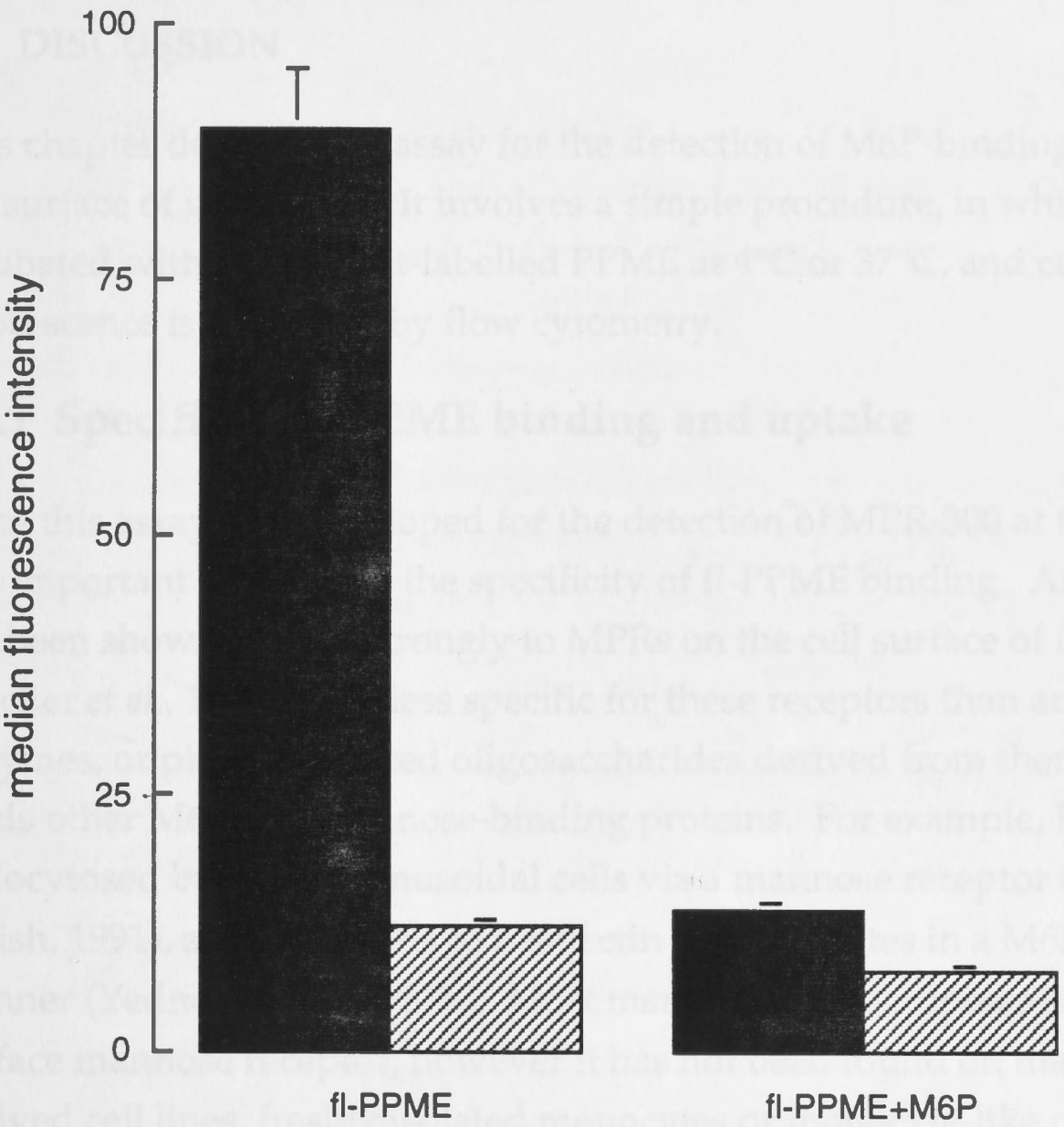


Fig. 2.11

Uptake (■) and binding (▨) of fl-PPME by non-adherent Clone 9 hepatocytes. Adherent cells were released with 0.1% EDTA and incubated with fl-PPME (50 µg/ml) for 3 h at 37°C Non-specific uptake/binding was determined by including 5 mM M6P in companion assays. Cell-associated fluorescence was determined by flow cytometry. All values are corrected for background autofluorescence. Values represent mean ± SEM (n=3).

concentration of fl-PPME used (50 µg/ml) was assumed sufficient to saturate binding and uptake by Clone 9 hepatocytes, as U937 cells incubated with FL-PPME at 50 µg/ml gave much higher fluorescence signals (up to 500-600 FIU).

2.4 DISCUSSION

This chapter describes an assay for the detection of M6P-binding proteins on the surface of intact cells. It involves a simple procedure, in which cells are incubated with fluorescent-labelled PPME at 4°C or 37°C, and cell-associated fluorescence is measured by flow cytometry.

2.4.1 Specificity of PPME binding and uptake

Since this assay was developed for the detection of MPR-300 at the cell surface, it is important to consider the specificity of fl-PPME binding. Although PPME has been shown to bind strongly to MPRs on the cell surface of fibroblasts (Fischer *et al.*, 1980c), it is less specific for these receptors than are lysosomal enzymes, or phosphorylated oligosaccharides derived from them, as it also binds other M6P and mannose-binding proteins. For example, PPME is endocytosed by splenic sinusoidal cells via a mannose receptor (Weston and Parish, 1991), and also binds to L-selectin on leukocytes in a M6P-dependent manner (Yednock *et al.*, 1987b). Most mammalian macrophages express a cell surface mannose receptor, however it has not been found on macrophage-derived cell lines, freshly isolated monocytes or monocyte-like cell lines such as U937 and HL-60 (Diment *et al.*, 1987; Kataoka and Tavassoli, 1985; Pimpaneau *et al.*, 1991; Roche *et al.*, 1990; Shepherd *et al.*, 1982). Thus, mannose binding is unlikely to contribute to the binding and uptake of fl-PPME described in this chapter, and in fact fl-mannan was not endocytosed by U937 cells, or by BRL-3A and Clone 9 hepatocytes.

M6P inhibited both binding and uptake of fl-PPME, confirming that binding was mediated by a M6P receptor. Three known cell surface proteins bind M6P, namely MPR-300, MPR-46 and L-selectin. It is unlikely that fl-PPME binds to cell surface MPR-46, as this receptor appears unable to bind or endocytose extracellular ligand (Stein *et al.*, 1987d). This was reinforced by the inability of both the McA 7777 hepatoma cell line and the $P388D_1$ macrophage cell line to bind and endocytose fl-PPME.

L-selectin is a lectin-like adhesion molecule which binds anionic sugar-bearing ligands such as PPME (Imai *et al.*, 1990; Yednock *et al.*, 1987b), and is constitutively expressed on the cell surface of unstimulated lymphocytes,

monocytes, neutrophils and other myeloid cells (Lewinsohn *et al.*, 1987; Rosen and Yednock, 1986). It is involved in adhesion of leukocytes to activated endothelium at inflammatory sites (Jutila *et al.*, 1991; Jutila *et al.*, 1989; Lewinsohn *et al.*, 1987; Watson *et al.*, 1991), and of lymphocytes to peripheral lymph node HEVs (reviewed by (Picker and Butcher, 1992)), and is rapidly down-regulated on activated cells (Jutila *et al.*, 1990; Jutila *et al.*, 1989; Kishimoto *et al.*, 1989a). Fl-PPME has been used extensively as a soluble model of it's natural ligand at a similar concentration range to that used in this study (Brandley *et al.*, 1987; Jutila *et al.*, 1990; Kansas *et al.*, 1991; Spertini *et al.*, 1991a; Spertini *et al.*, 1991b; Watanabe *et al.*, 1992; Yednock *et al.*, 1987a). It is possible that the fl-PPME binding assay described in this chapter could detect cell surface L-selectin, as well as MPR-300, on leukocytes expressing both molecules. While L-selectin has not been reported on U937 cells, this is a monocyte-like cell line and it may well be expressed and contribute to binding of fl-PPME.

Measurement of fl-PPME uptake, rather than binding, biases the assay in favour of MPR-300, as L-selectin has not been implicated in endocytic uptake of carbohydrate ligands. In the presence of cell surface MPR-300, any binding of fl-PPME to L-selectin would be masked by fluorescence due to MPR-mediated endocytosis. Other characteristics of fl-PPME uptake were also consistent with the known behaviour of MPR-300. Linear uptake of fl-PPME over a prolonged period, indicating an undiminished supply of unoccupied receptors, is consistent with recycling of MPR-300. Inhibition of uptake by chloroquine is compatible with the demonstration by (Gonzalez-Noriega *et al.*, 1980) that preventing ligand release in the prelysosomal compartment depletes the cell of unoccupied MPR-300, and prevents further endocytosis of exogenous ligands. Inhibition of fl-PPME uptake by various phosphosugars was also consistent with the known specificity of MPR-300. Uptake by U937 cells was strongly inhibited by M6P and F1P, both potent inhibitors of ligand binding to MPR-300 and MPR-46 (Kaplan *et al.*, 1977a; Tong and Kornfeld, 1989). The concentration of M6P required to produce a 50% inhibition of uptake was approximately 0.4 mM, compatible with the binding specificity of MPR-300 (0.95 mM) (Distler *et al.*, 1991) . Low inhibition by F6P, G6P, Gal6P and M1P were also consistent with binding to MPR-300 (Kaplan *et al.*, 1977a). In summary, uptake of fl-PPME by U937 cells appears to be mediated by MPR-300. This assay may, however, be unsuitable for detecting small amounts of MPR-300 on cells which also express high levels of L-selectin.

2.4.2 Detection of MPR-300 on the surface of U937 cells by binding and uptake of fl-PPME

Fl-PPME binding to the surface of cells at 4°C was saturable and M6P-specific, providing a direct, although insensitive, measure of MPR-300 at the cell surface. Receptor-mediated endocytosis of fl-PPME at 37°C was far more sensitive, as it exploited the continuous recycling of MPR-300. As ligand internalized by MPR-300 is delivered to lysosomes while empty receptors recycle back to the cell surface, large amounts of fl-PPME accumulated within the cell over time, producing a very high fluorescence intensity, limited only by the incubation time and the amount of fl-PPME supplied. As PPME must initially bind to receptors at the cell surface, this assay is totally dependent on the presence of cell surface MPR-300, and is, in effect, a cumulative measure of total cell surface expression over the time of the assay.

Endocytosis of fl-PPME by cells thus provides a useful and sensitive assay for the presence of MPR-300 at the cell surface. It should be noted that the rate of endocytosis contributes to the extent of fl-PPME accumulation also, so PPME uptake does not provide a quantitative measure of cell surface MPR-300. Instead, it indicates the presence of MPR-300 on the plasma membrane of a given cell type, with greater sensitivity than PPME binding. This assay does not lend itself easily to mathematical analysis of binding and uptake (Spertini *et al.*, 1991a), as PPME is multivalent. However, it should be useful for comparisons between different cell types or treatments.

2.4.3 Selection of a suitable rat cell line for antibody screening

A rat cell line expressing MPR-300 and MPR-46 on the cell surface was required for screening hybridoma supernatants for their ability to bind to cell-associated MPRs. As liver tissue and cell lines have been used in many studies of MPR expression, two rat hepatocyte cell lines were examined using the PPME uptake assay. Clone 9 and BRL-3A cells are both reported to express MPR-300 on the cell surface (Brown *et al.*, 1984; Clairmont and Czech, 1991). MPR-46 distribution has been studied in Clone 9 cells, however cell surface expression was not noted (Matovcik *et al.*, 1990). As the two receptors have been reported to have similar subcellular distributions in other cell types (Bleekmolen *et al.*, 1988), MPR-46 may also be present on the Clone 9 cell surface. Expression of MPR-46 has not been examined in BRL-3A cells.

Comparison of MPR-300 cell surface expression on these two cell lines, using the fl-PPME uptake assay, showed that Clone 9 cells expressed far more MPR-300 on the cell surface than did the BRL-3A cell line. The Clone 9 cell line was thus selected for screening of hybridoma supernatants.

2.4.4 PPME binding and uptake as a measure of MPR function

One aim of this study was to develop a screening assay to identify antibodies able to interfere with ligand binding to cell surface MPR-300. The fl-PPME binding and uptake assays both detect MPR-300 on the basis of its ability to bind and endocytose M6P-bearing ligands, thus both assays should be sensitive to inhibition of ligand binding. The assay procedure is suitable for use as a screening assay, as it is very simple, and the incubation of cells with fl-PPME can be done in a microtitre plate, a format which facilitates the handling of large numbers of samples. Fl-PPME binding to the cell surface may limited in this application by its low sensitivity. Fl-PPME uptake by cells is more sensitive than binding, and so provides a larger window for detecting inhibition of ligand binding, with the disadvantage that exogenous antibody will be rapidly internalized and depleted from the medium. A high initial concentration of antibody will therefore be required to ensure that there is sufficient to bind fresh MPR-300 as it arrives at the cell surface over the duration of the incubation period.

2.5 SUMMARY

Lysosomal enzymes expressed at the cell surface in association with MPR-300 are proposed to contribute to the invasive capacity of activated T lymphocytes by participating in basement membrane degradation. In order to examine the consequences of specifically inhibiting the enzyme-MPR interaction on the ability of T cells to degrade basement membrane components *in vitro* and initiate an inflammatory response in the rat model EAE, monoclonal antibodies were raised against MPR-300. This chapter describes a simple ligand binding assay for MPR-300, developed to (a) identify a suitable rat cell line for screening hybridoma supernatants for antibodies specific for the extracellular portion of MPR-300; and (b) facilitate screening of antibodies for their ability to inhibit the binding of M6P-bearing ligands to MPR-300.

The human monocytic cell line U937, known to express both MPR-300 and MPR-46 on the plasma membrane, was used to determine the characteristics of PPME binding and endocytosis. Fluorescein-labelled PPME bound saturably to

the cell surface at 4°C, in a M6P-inhibitable manner. Cell-associated fluorescence was quantitatively low, however, and endocytosis of PPME provided a more sensitive indicator of cell surface binding sites. PPME uptake at 37°C was dose saturable at any given incubation time, and inhibitable by M6P.

Several characteristics of PPME uptake correlate with the known behaviour of MPR-300, suggesting that endocytosis is mediated by this receptor: (a) two cell lines which express MPR-46 but are deficient in MPR-300 did not bind or endocytose PPME; (b) PPME uptake by cells was limited only by the amount supplied, consistent with recycling of MPR-300; (c) internalized PPME is compartmentalized, consistent with ligand release in the prelysosomal compartment, and subsequent delivery to lysosomes; (d) PPME uptake was inhibited by chloroquine treatment, which prevents dissociation of the MPR-300-ligand complex in intracellular acidified compartments, depleting the cell of unoccupied receptors; and (e) PPME uptake was inhibited by M6P and fructose-1-phosphate, but not by galactose-1-phosphate, mannose-1-phosphate or mannose. Uptake of PPME generated a far greater fluorescent signal than produced by cell surface binding, thus endocytosis of fluorescein-labelled PPME provides a sensitive assay for the presence of MPR-300 at the cell surface.

Two rat cell lines, Clone 9 and BRL-3A hepatocytes, were shown to endocytose PPME in a M6P-inhibitable manner. As uptake by Clone 9 cells was 35-fold greater than by BRL-3A cells, this cell line was selected for screening hybridoma supernatants for antibodies specific for the extracellular domain of MPR-300. As McA RH7777 cells did not internalize PPME, they were selected as a negative control for MPR-300, and for detecting antibodies with specificity for MPR-46.

Both PPME binding and uptake were suitable assays for screening antibodies for their ability to inhibit M6P-mediated ligand binding. Both detect cell surface MPR-300 on the basis of its ability to bind a M6P-bearing ligand, and in addition, the assay procedure is simple and conducive to handling large numbers of samples.

CHAPTER 3

PREPARATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR MPR-300

3.1 INTRODUCTION

Parish and coworkers have proposed that lysosomal enzymes, attached to the cell surface of extravasating leukocytes by MPRs, participate in degrading the subendothelial basement membrane. As activated T lymphocytes are required for the passive transfer of EAE, they further proposed that cell surface expression of lysosomal enzymes may be enhanced by cellular activation, thus contributing to an increased invasive capacity. As described in Section 1.14, such as increase could be mediated by an activation-induced redistribution of the existing MPR pool, such that MPR-300 expression is increased at the cell surface, thus increasing the number of binding sites for extracellular lysosomal enzymes. An increase in intracellular expression of MPR-46 could also contribute, as this receptor is directly involved in the secretion of lysosomal enzymes, and so could increase their concentration in the extracellular space. The aim of the experiments described in this chapter was to produce monoclonal antibodies (mAbs) specific for MPR-300 and MPR-46, to enable the examination of (a) the cellular distribution of MPR-300 and MPR-46 in resting and activated T cells; and (b) the consequences of neutralizing the binding of lysosomal enzymes to cell surface MPR-300.

The effect of inhibiting lysosomal enzyme binding to cell surface MPR-300 has been partially explored using M6P as a competitive inhibitor. M6P binds to both purified MPR-300 (Tong *et al.*, 1989) and MPR-46 (Tong and Kornfeld, 1989), and is a potent inhibitor of lysosomal enzyme uptake by intact cells (Kaplan *et al.*, 1977a; Kaplan *et al.*, 1977c; Sando and Neufeld, 1977; Ullrich *et al.*, 1978). M6P also inhibited the heparanase activity of PMA-stimulated human peripheral blood lymphocytes in an *in vitro* ECM degradation assay (Bartlett *et al.*, 1995a), and diminished lesion formation in adoptively transferred EAE and adjuvant arthritis in rats (which are initiated by entry of activated T cells into the CNS and joints respectively) (Willenborg *et al.*, 1989b; Willenborg *et al.*, 1992). These three studies demonstrate a positive correlation between inhibition of lysosomal enzyme binding to MPRs and loss of T cell degradative activity. However, M6P could conceivably act by some means other than disrupting the MPR-lysosomal enzyme complex. Thus, as well as providing reagents which could be used to compare the cellular distribution of MPRs in resting and activated cells, an aim of generating mAbs to these receptors was to provide a means of specifically inhibiting the interaction between cell surface MPR-300 and extracellular lysosomal enzymes, the effect of which could then

be examined on the ability of T cells to degrade basement membrane *in vitro* and induce EAE *in vivo*.

Several groups have prepared polyclonal antibodies which block ligand binding to human MPR-300, and prevent binding and endocytosis of extracellular lysosomal enzymes by fibroblasts (Creek and Sly, 1983; von Figura *et al.*, 1984; Gartung *et al.*, 1985; Kiess *et al.*, 1989). Antibodies prepared by another group did not strongly inhibit ligand binding to MPR-300, but instead depleted the receptor from the cell surface. Internalized MPR-300 normally recycles to the Golgi/TGN or back to the plasma membrane, however cross linking of cell surface receptors by polyclonal antibodies, or by Fab fragments linked by a secondary antibody, caused their accumulation in an unidentified compartment from which they did not recycle but were ultimately degraded (Nolan *et al.*, 1987). Antibodies capable of preventing the binding of extracellular lysosomal enzymes to cell surface MPR-300 have thus been described for the human receptor. This study aimed to produce similarly effective antibodies to rat MPR-300, so that their effect on passively-induced EAE could be examined. MAbs were produced in preference to polyclonal antisera due to their greater specificity and reproducibility. This chapter describes the preparation of mAbs specific for rat MPR-300, and an assessment of their potential for neutralizing the binding of lysosomal enzymes to this receptor.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Purification of MPR from rat liver

3.2.1.1 Preparation of rat liver extract

A mixture of MPR-300 and MPR-46 was purified from rat liver using a modification of the method of (Li *et al.*, 1989), who purified the two MPRs from bovine testes. Rats were killed by CO_2 asphyxiation and the livers removed, rinsed in cold PBS, weighed and stored at -20°C. A tissue mass of 130-150 g per batch was found most convenient. Frozen livers were thawed and blended thoroughly , adding sufficient 1 mM PMSF/PBS to liquify the tissue. This and all following steps was done at 4°C. The homogenate was centrifuged for 15 min at 21, 000xg, and the pellet washed in distilled water containing 1 mM PMSF. The pellet was resuspended in Solubilizing Buffer (50 mM imidazole (pH 6.5), 150 mM sodium chloride, 1% Triton X-100, 0.5 mM PMSF) and agitated overnight. The suspension was centrifuged for 60 min at 100, 000xg

and the supernatant passed through a single thickness of Whatman No. 1 filter paper. 1 M $MnCl_2$ stock solution was added to give a final concentration of 10 mM $MnCl_2$, and the supernatant immediately subjected to affinity chromatography on PMP-Sepharose 4B, as described in Section 3.2.1.3.

3.2.1.2 Preparation of PMP-Sepharose

The affinity matrix was prepared by coupling an arylamino derivative of pentamannose phosphate (PMP) to cyanogen bromide-activated Sepharose 4B. PMP was isolated from the exopolysaccharide secreted by the yeast *Pichia holstii* (strain ATCC 13689), and was provided by Dr Susan Weston (Manchester University, UK). It was coupled to 2-(4-aminophenyl) ethylamine using a modification of the method of (Jeffery *et al.*, 1975). PMP (600 mg) was added to 2-(4-aminophenyl) ethylamine (3 ml) in a 20 ml round-bottomed flask with a drying tube (half soda lime, half calcium chloride) attached,and stirred at 37°C until the PMP dissolved to form a viscous solution (5 days). Absolute ethanol (3 ml) was added, followed immediately with sodium borohydride (72 mg) in ethanol (6 ml), and stirred at 4°C for 48 h, with a drying tube attached. The mixture was diluted with cold distilled water (24 ml) and the pH adjusted to 5.6 with glacial acetic acid. Ethanol and water were evaporated under reduced pressure and the PMP derivative precipitated from the residue with 4 volumes of ethanol. The precipitate was isolated by centrifugation, resuspended in 0.5 M sodium chloride in PBS and reprecipitated with four volumes of ethanol. This step was repeated a further three times. The precipitate was then dissolved in PBS, reprecipitated with four volumes of ethanol, and dissolved in distilled water and lyophilised, yielding 490 mg. Amine determination (A_{285}) indicated that 42% of the PMP was derivatized. The product was stored in 50 mg portions under nitrogen in a dessicator at -20°C.

The arylamino PMP derivative (52 mg; 21.6 µmol) was coupled to 7 ml of swollen cyanogen bromide-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) following the manufacturer's instructions. Coupling efficiency was 96%.

3.2.1.3 Purification of MPRs by affinity chromatography

The rat liver extract (Section 3.2.1.1) was pre-adsorbed on a Sepharose 4B column (2.5 x 4 cm), equilibrated with Binding Buffer I (50 mM imidazole (pH 6.5), 0.5 M sodium chloride, 10 mM $MnCl_2$, 0.5 mM PMSF, 0.05% Triton X-100).

The eluate was then applied to a PMP-Sepharose column (1.5 x 5.5 cm), also equilibrated with Binding Buffer I, and allowed to flow by gravity feed. Unbound protein was removed by washing with 500 ml of Binding Buffer I. Triton X-100 was exchanged for octylglucoside by washing with Binding Buffer II (50 mM imidazole (pH 6.5), 0.5 M sodium chloride, 10 mM $MnCl_2$, 0.5 mM PMSF, 25 mM octylglucoside). MPR-300 and MPR-46 were eluted with 40 ml of either Eluting Buffer I (100 mM sodium acetate, pH 4.5, 0.5 M sodium chloride, 10 mM $MnCl_2$, 25 mM octylglucoside, 0.5 mM PMSF) or Eluting Buffer II (20 mM M6P, 0.5 M sodium chloride, 10 mM $MnCl_2$, 25 mM octylglucoside, 0.5 mM PMSF), and 1 ml fractions collected. Fractions eluted with Eluting Buffer I were immediately neutralized with 0.5 M imidazole (pH 6.5). Fractions were analysed by SDS-PAGE and the protein bands visualized by silver staining (Section 3.2.1.4). MPR-containing fractions were pooled and concentrated by centrifugation in Centricon-10 microconcentrators (Amicon) at 3000xg until the volume was below 200 µl. The solution was diluted with 25 mM octylglucoside in PBS and reconcentrated. This step was repeated, and the concentrated MPR solution stored at -20°C.

The PMP-Sepharose column was regenerated after use by washing with 100 mM glycine-HCl, pH 2.5, then three alternating washes with 0.1 M boric acid, 0.5 M sodium chloride (pH 8) and 0.1 M acetic acid, 0.5 M sodium chloride (pH 4.0).

3.2.1.4 SDS-PAGE

Column fractions were analysed by one dimensional 10% SDS-PAGE according to the method of Laemmli (1970). Samples (30 µl) were mixed with an equal volume of 2x sample buffer (125.mM Tris, 100 mM dithiothreitol, 160 mM SDS, 60% v/v glycerol, pH 6.8) and boiled for 5 min before loading onto the gel. Proteins were electrophoresed at a constant current of 20 mA for 3 h. Gels were silver stained using the method of Blum *et al.* (1987) and dried onto blotting paper under vacuum in a gel dryer at 80°C for 1 h. Molecular weight markers used were rabbit skeletal muscle myosin MW 200, 000; E. coli β-galactosidase MW 116, 250; rabbit muscle phosphorylase b MW 97, 400; bovine serum albumin MW 66, 200; hen egg white ovalbumin MW 45, 000; bovine carbonic anhydrase MW 31, 000; soybean trypsin inhibitor MW 21, 500; and hen egg white lysosyme MW 14, 400 (Biorad Laboratories, Richmond, CA).

3.2.2 Immunization of mice

The MPR preparation (5-100 µg diluted in 200 µl of saline) was mixed with an equal volume of Freund's complete adjuvant under sterile conditions and injected subcutaneously into four sites on the flanks of 8 week old female BALB/c mice (50 µl per site). Preimmune serum was obtained before the first injection. After 4 weeks, mice were boosted with 5-100 µg of the MPR preparation in 200 µl of saline, mixed with an equal volume of Freund's incomplete adjuvant. Ten days later, blood was collected from a lateral tail vein and assayed for the presence of antibodies specific for MPR (Section 3.2.8.1). A second boost was given three days before fusion.

Antigen was prepared by placing the adjuvant in a small glass bottle, held at an angle with plasticine. The MPR stock solution (in 25 mM octylglucoside/PBS) was diluted with saline and added dropwise to the adjuvant, mixing well with a glass syringe until an emulsion formed.

3.2.3 Production of antibody-secreting hybridomas

3.2.3.1 Preparation of a single cell suspension from mouse spleen

Three days after the final immunization, mice were killed by cervical dislocation and the spleen removed and washed in serum-free DMEM. This and all following steps were performed under sterile conditions. A cell suspension was prepared by finely mincing the spleen with curved surgical scissors and gently pressing the tissue through a fine wire mesh into serum-free DMEM. The cell suspension was transferred to a 50 ml centrifuge tube, leaving behind any large pieces of tissue. Lymphocytes were counted by removing a 10 µl sample of the spleen cell suspension and lysing red blood cells with 40 µl of 0.83% ammonium chloride for 3 min at 37°C. The unlysed cells were counted using a haemocytometer and viability assessed by trypan blue exclusion. Spleens contained approximately 2.5×10^8 cells. 5×10^6 cells were reserved as tissue culture controls to moniter death of unfused spleen cells.

3.2.3.2 Preparation of myeloma cells

The fusion partner was the murine myeloma cell line P3-NS1/1-Ag4-1, which does not secrete immunoglobulin, but does synthesize light chains, which can be incorporated into immunoglobulins in hybrid cells. On average, one immunoglobulin molecule in four produced by a hybrid cell will be completely derived from the spleen cell partner (Galfre and Milstein, 1981; Kohler and

Milstein, 1976). Myeloma cells were harvested from a logarithmic phase culture, and viability determined by trypan blue exclusion. $5x10^6$ cells were reserved as tissue culture controls to moniter death of unfused myeloma cells.

3.2.3.3 Fusion of spleen and myeloma cells

The myeloma and spleen cell suspensions were mixed such that the ratio of spleen to myeloma cells was 5:1, and centrifuged in a 50 ml plastic tube at 200xg for 5 min. The supernatant was discarded and the cell pellet resuspended in serum-free DMEM and recentrifuged, to ensure even distribution of the fusion partners. The supernatant was again discarded, and the pellet loosened. Fusion of spleen and myeloma cells was mediated by polyethylene glycol (PEG). A 40% PEG solution (2 ml) was warmed to 37°C and added to the cell pellet dropwise over exactly 30 s, while constantly agitating the tube. When PEG addition was complete, the cells were resuspended over 30 s by gently bubbling air through a 5 ml pipette. The cell suspension was then allowed to stand for 30 s, before being gently diluted by dropwise addition of 5 ml of serum-free DMEM over 2 min, followed by another 5 ml added all at once. The cells were allowed to stand for 3 min, then centrifuged at 500xg for 5 min. The cell pellet was resuspended gently in 190 ml of medium containing HAT (Table 3.1). Aliquots (100 µl) of the cell suspension were placed in the wells of flat-bottomed 96-well tissue culture plates (Linbro, ICN Biomedicals, Costa Mesa, CA), using a multichannel pipette with wide bore tips. Feeder cells were not required as conditioned medium from human umbilical vein endothelial (HUVE) cells was included in the medium (Astaldi, 1980). Reserved spleen and myeloma cells were resuspended at $1x10^6$ cells/ml in HAT medium, and similarly aliquoted into a 96-well plate. These were observed after 7 days to ensure the deaths of both parent cell types.

3.2.4 Maintenance of hybridomas

3.2.4.1 Initial cell culture

Cultures were incubated in a humidified (90%) atmosphere of 10% CO_2 in air at 37°C. Plates were left undisturbed until the 7th day post fusion, when 100 µl of HT medium (Table 3.1) was added to each well. Between 7 and 14 days after fusion, all wells were examined for colony growth using an inverted microscope (Olympus Optical Co. Ltd., Tokyo, Japan). On day 14, 150 µl of medium was transferred from wells with colony growth into sterile 96 well plates and stored at 4°C for use in screening assays. Medium from wells with

Table 3.1

Constituents of hybridoma culture media.

HAT medium[a]

15% v/v hybriserum

10% v/v endothelial cell conditioned medium[b]

0.1% v/v 2-mercaptoethanol

2.5 mg/L amphotericin

13.6 mg/L hypoxanthine

3.9 mg/L thymidine

72 µg/L aminopterin (omitted from HT medium)

Freezing medium[a]

20% v/v hybriserum

0.1% v/v 2-mercaptoethanol

2.5 mg/L amphotericin

6% v/v dimethylsulfoxide (DMSO)

[a] All media were based on Dulbecco's Modified Eagle Medium (DMEM; #430-1600, Gibco, Gaithersburg, MD). Medium was supplemented with 3.7 g/L sodium bicarbonate, 200 mg/L neomycin sulfate, 200 mg/L streptomycin sulfate, and 120 mg/L penicillin were added to all media.

[b] Provides growth-promotion activity for hybridoma cells, and substitutes for feeder cells (Astaldi, 1980)

no colony growth was discarded. Wells with large amounts of cell growth were subcultured: cells were resuspended in 150 µl of HT medium and 100 µl was transferred to a new well in a flat-bottomed 96-well tissue culture plate (Linbro, ICN Biomedicals, Costa Mesa, CA). Fresh HT medium (150 µl) was added to all colony-containing wells.

3.2.4.2 Expansion of cultures into 24-well plates

Culture supernatants collected between 2 and 4 weeks post fusion were tested for the presence of MPR-specific antibodies using the DELFIA screening assay (Section 3.2.8.1). Cultures which gave a strong positive response were expanded into 24-well plates, so as to prepare a reserve of frozen cells in case cloning was unsuccessful. Spent medium was removed from wells and cells were resuspended in fresh HT medium (100 µl). The cell suspension was transferred to one well of a 24-well plate, and diluted to 1 ml with fresh medium. HT medium (150 µl) was added to the original well to allow regrowth of any remaining cells.

3.2.4.3 Freezing hybridoma cultures

96-well plates containing subcultured cells were eventually frozen. Spent medium was removed from all wells and replaced with 50 µl of ice-cold freezing medium (Table 3.1). Each plate was wrapped first with plastic wrap, then with bubble-wrap and placed immediately at -70°C.

Cells transferred to 24 well plates were frozen when the culture was expanded into at least 4 wells. Healthy cells were harvested by centrifugation at 4°C, resuspended in chilled freezing medium, and transferred in 1 ml aliquots to plastic 2 ml cryotubes (Nunc, Denmark). Cryotubes were frozen at -20°C for 2 h, then at -70°C overnight. Frozen tubes were placed in liquid nitrogen for long term storage.

3.2.4.4 Thawing frozen cells

Frozen cells were thawed into 10 ml HT medium, washed once and counted. Cells were resuspended at a density of 2-4 x 10^6 cells/ml and aliquots (100 µl) were placed in the wells of a U-bottomed 96-well tissue culture plate (Linbro, ICN Biomedicals, Costa Mesa, CA). After 7 days, the cells were screened for antibody production (Section 3.2.8.1) and expanded into 24-well plates.

3.2.4.5 Collection of hybridoma culture supernatants

Hybridomas were grown in either 24-well tissue culture plates or tissue culture flasks of varying sizes. Cultures were grown until the cells died, and the supernatant was collected. Debris was removed by centrifugation at 1000xg for 10 min. Small volumes were passed through sterile Acrodisc PF syringe filters (Gelman Sciences, Ann Arbor, MI) and larger volumes through 0.2 µm bottle filters (Costar, Cambridge, MA). Filtered supernatants were stored in sterile plastic containers at 4°C.

3.2.5 Cloning by limiting dilution

A suspension of viable hybridoma cells (10 cells/ml) was prepared by serial dilution in 48 ml of HT medium. Aliquots (100 µl) were placed in the wells of 96-well round-bottomed tissue culture plates (Linbro, ICN Biomedicals, Costa Mesa, CA), giving an average of 1 cell/well. As previously, feeder cells were replaced by the inclusion of conditioned medium from HUVE cells in the culture medium. The plates were incubated at 37°C (90% humidity, 10% CO_2 in air) and left undisturbed for 5 days. All plates were then examined for signs of colony growth, and wells with a single small colony were noted. Wells with two or more colonies were disregarded, as were wells containing large single colonies which could have resulted from the merging of several rapidly growing smaller ones. When growth of single colonies covered about 1/3 to 1/2 of the well, cells were subcultured 1:2 into flat-bottomed 96 well plates (Linbro, ICN Biomedicals, Costa Mesa, CA). When both wells reattained this level of growth, cells were expanded into 24-well plates.

3.2.6 Production of ascites fluid containing monoclonal antibodies

The hybridoma 1G7/9H4 was grown in HT medium in 175 cm^2 tissue culture flasks. Cells were harvested by centrifugation, washed with sterile PBS and resuspended at a concentration of 2.5 x 10^6 cells/ml. Outbred Swiss nude mice, injected five to ten days earlier with 1 ml of pristine (2,6,10,14-tetramethylpentadecane), were injected i.p. with 2 ml of cells. Two to three weeks later, mice were killed and peritoneal fluid harvested. Ascites from twenty mice were pooled and delipidated by passing through glass wool.

3.2.7 Purification of IgG_1 from ascites fluid

Antibodies (IgG_1) were purified from 1G7/9H4 ascites by affinity chromatography using the Affi-Prep Protein A MAPS II Kit (Bio-Rad Laboratories, North Ryde, Australia), following the procedure recommended in the manufacturers instructions.

3.2.8 Screening hybridoma culture supernantants: antibody detection assays

3.2.8.1 Antibody detection by Dissociation Enhanced Lanthanide Fluoroimmunoassay (DELFIA)

The MPR preparation (in 25 mM octylglucoside/PBS) was diluted to 2.5 µg/ml with coating buffer (2.86 g/l Na_2CO_3, 7.56 g/l $NaHCO_3$). Round-bottomed, polyvinylchoride (PVC) microtitre plates (Dynatech Laboratories Inc., Alexandria, VA) were coated overnight at 4°C with dilute MPR (100 µl/well). Control wells were incubated with coating buffer alone. Plates were blocked with 3% BSA in PBS for 2 h at 37°C (200 µl/well) and were washed three times with 0.05% Tween 20 in PBS (PBST) after this and all subsequent steps. All subsequent dilutions were in 1% BSA/PBST, except where otherwise stated, and all incubations were at room temperature. Test antibody (either undiluted culture supernatant, supernatant diluted in HT medium, or antiserum diluted in 1% BSA/PBST) was added (50 µl/well) and incubated for 2 h, followed by a 1:1000 dilution of biotinylated rabbit- antimouse Ig $F(ab')_2$ fragment (Dakopatts, Denmark) for 1 h (50 µl/well), and a 1:1000 dilution of DELFIA Europium-Labelled Streptavidin (Wallac Oy, Turku, Finland) for 1 h (75 µl/well). DELFIA Enhancement Solution (Wallac Oy, Turku, Finland) was added and incubated for 5 min (200 µl/well). Fluorescence was measured using a 1234 DELFIA Research Fluorometer (Pharmacia, Uppsala, Sweden) and data analysed using Multicalc software. Specific antibody binding was calculated by subtracting fluorescence due to antibody binding to an uncoated well from fluorescence due to binding to a MPR-coated well.

3.2.8.2 Antibody detection by immunofluorescent flow cytometry

3.2.8.2.1 Antibody binding to Clone 9 hepatocytes

Adherent Clone 9 hepatocytes were detached with 0.1% EDTA in PBS, and resuspended in 10% FCS/RPMI medium at a density of $1x10^7$ cells/ml. Cells were treated with hybridoma supernatant, washed and incubated with a

saturating concentration of an affinity-isolated sheep anti-mouse Ig $F(ab')_2$ fragment, directly conjugated to fluorescein (Silenus, Hawthorne, Australia). Incubations were in 96-well V-bottomed plastic plates, using $2x10^5$ cells/well in 40 µl volumes. All incubations were for 30 min on ice, and cells were washed three times with 10% FCS/RPMI medium between the two incubations. The final incubation was followed by washing twice with 10% FCS/RPMI and once with PBS. Cells were fixed with 1% paraformaldehyde/PBS, pH 7.4 and analysed for fluorescence by flow cytometry (Section 2.2.3). Samples treated with hybridoma supernatants were compared with control samples incubated with an irrelevant hybridoma culture supernatant, specific for human endothelial cell surface antigens.

3.2.8.2.2 Antibody binding to U937 cells

U937 cells were harvested by centrifugation and resuspended in 10% FCS/RPMI medium at a density of $5x10^6$ cells/ml. Cells were treated with hybridoma supernatant, washed and incubated with a saturating concentration of a Protein A-fluorescein conjugate (100 µg/ml) (Pharmacia, Uppsala, Sweden). Incubations were in 96-well V-bottomed plastic plates using 1×10^5 cells/well in 40 µl volumes. All incubations were for 30 min on ice, and cells were washed three times with 10% FCS/RPMI medium between the two incubations. The final incubation was followed by washing twice with 10% FCS/RPMI and once with PBS. Cells were fixed with 1% paraformaldehyde/PBS, pH 7.4 and analysed for fluorescence by immunofluorescent flow cytometry (Section 2.2.3). Samples treated with hybridoma supernatants were compared with control samples incubated with an irrelevant hybridoma culture supernatant, specific for human endothelial cell surface antigens.

3.2.9 Immunoprecipitation by hybridoma culture supernatants

3.2.9.1 Biotinylation of the affinity purified MPR preparation

MPR stock solution was diluted to 200 µg/ml with 100 mM $NaHCO_3$ buffer, pH 8.5, containing 25 mM octylglucoside. A 20 mM stock solution of sulfo-S-biotin (Pierce, Rockford, IL) was prepared in the same buffer, and sufficient added to the MPR solution to give a final concentration of 2 mM. The reaction mixture was incubated at 4°C for 2 hours. Free biotin was removed by concentrating in a Centricon-10 microconcentrator and rediluting with 25 mM octylglucoside/PBS. This procedure was repeated twice more. The

biotinylated protein was diluted to a concentration of 100 µg/ml and stored in 100 µl aliquots at -20°C.

3.2.9.2 Immunoprecipitation of biotinylated MPR

Protein G-Sepharose (20 µl packed volume) (Pharmacia, Uppsala, Sweden) was washed with 0.5% Triton X-100/PBS (TPBS) and incubated with 300 µl of hybridoma culture supernatant containing 0.5 % Triton X-100, for 2 hours at 4°C. The beads were centrifuged and washed four times with TPBS. Biotinylated MPR was diluted to 3.3 µg/ml with 0.1% BSA/TPBS and preadsorbed to washed protein G-Sepharose overnight at 4°C. The preadsorbed MPR (300 µl) was incubated with the antibody-coated protein G-Sepharose for 4.5 h at 4°C. The beads were centrifuged, and washed six times with TPBS. Reduced Sample Buffer (30 µl) (Section 3.2.1.4) was added to each sample and boiled for 1 minute. The beads were pelleted by centrifugation and the supernatants analysed by SDS-PAGE (Section 3.2.1.4). Protein was electroblotted onto a Hybond-C Super transfer membrane (Amersham, Buckinghamshire, England) at a constant current of 120 mA for 90 min. The buffer for both anode and cathode contained 39 mM glycine, 48 mM Tris, 0.0375% w/v SDS and 20% methanol. The membrane was air-dried, then blocked with 3% BSA/0.05% Tween-20/PBS (PBST) for 1 h, washed with PBST (2 rinses, 2x5 minutes) and probed with streptavidin-horseradish peroxidase conjugate (1/1000 dilution in 0.1% BSA/PBST) (Amersham, Buckinghamshire, England) for 1 hour at RT. The membrane was washed with PBST (2 rinses, 1x15 minutes, 4x5 minutes). Biotinylated protein was detected using the ECL detection reagent (Amersham, Buckinghamshire, England).

3.2.10 Determination of antibody isotype by ELISA

Four PVC microtitre plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with affinity-purified goat anti-mouse Ig antibodies (Southern Biotechnology, Birmingham, AL) overnight at 4°C. Antibodies were specific for either mouse IgM, IgG_1, IgG_{2a} or IgG_{2b}. They were diluted in coating buffer (2.86 g/L Na_2CO_3, 7.56 g/L $NaHCO_3$) to concentrations of 0.5 µg/ml (anti-IgM) and 0.2 µg/ml (anti-IgG_1, IgG_{2a} and IgG_{2b}), and used at 100 µl/well. Plates were blocked with 3% BSA/PBS for 2 h at 37°C (200 µl/well) and washed three times with 0.05% Tween 20 in PBS (PBST) after this and all subsequent steps. All subsequent dilutions were in 1% BSA/PBST, except where otherwise stated, and all incubations were at room temperature. Hybridoma culture supernatant was added (50 µl/well) and incubated for 2 h,

followed by 50 µl/well of a biotinylated isotype-specific goat anti-mouse Ig antibody for 1 h (1 µg/ml for anti-IgM and IgG2a, 0.25 µg/ml for anti-IgG1, 2 µg/ml for anti-IgG_{2b}) (Southern Biotechnology, Birmingham, AL). Wells were incubated with 75 µl streptavidin-horseradish peroxidase conjugate (Amersham, Buckinghamshire, England) for 1 h. Substrate solution (1 mg/ml 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; 0.03% hydrogen peroxide in 0.1 M sodium citrate buffer, pH 4.5) was added (100 µl/well) and incubated until colour developed. The reaction was stopped with 0.2 M citric acid (100 µl/well) and absorbance at 405 nm (reference 490 nm) measured immediately using a microplate reader (Molecular Devices). Data was analysed using Softmax software.

3.2.11 Effect of antibodies on ligand binding to MPR

3.2.11.1 Lysosomal enzyme binding to purified MPR

3.2.11.1.1 Secretion of newly synthesized lysosomal enzymes by Clone 9 hepatocytes

Clone 9 hepatocytes were subcultured at a ratio of 1:2, and 1M ammonium chloride added to the medium to give a final concentration of 10 mM. Cells were cultured for 60 h, and the lysosomal enzyme-enriched culture medium collected and frozen in 1 ml aliquots.

3.2.11.1.2 Lysosomal enzyme binding to purified MPR

PVC microtitre plates were coated overnight at 4°C with 100 µl/well of sheep anti-mouse Ig antibody (Silenus, Hawthorne, Australia), diluted to 10 µg/ml in coating buffer (2.86 g/L Na_2CO_3, 7.56 g/L $NaHCO_3$). Wells were blocked with 3% BSA/PBS for 1.5 h at 37°C (200 µl/well), and washed three times with 0.05% Tween 20 in PBS (PBST) after this and all subsequent steps. All dilutions were in 1% BSA/PBST, except where otherwise stated, and all incubations were at room temperature. Culture supernatant from the hybridoma 5G5/17B5 (50 µl/well) was added and incubated for 1 h, followed by 50 µl of dilute MPR preparation (2.5 µg/ml) for 2 h. Lysosomal enzyme-enriched Clone 9 culture medium was added (50 µl/well) and incubated for 2 h. 10 mM mannose or 10 mM M6P was included in control wells. Wells were incubated overnight at 37°C with 200 µl of β-glucuronidase substrate solution (10 µl of 100 µM 4-methylumbelliferyl-β-D-glucuronide, 190 µl of 20 mM sodium acetate, pH 5.0). The contents of each well was transferred to a 96-well MicroFluor microtitre plate (Dynatech Laboratories Inc., Alexandria, VA). β-Glucuronidase activity

was estimated in fluorescence intensity units (FIU) using an automated fluorescence plate reader (MicroFluor Reader, Dynatech Laboratories Inc., Alexandria, VA).

3.2.11.1.3 The effect of antibodies on binding of lysosomal enzymes to purified MPR

The ability of hybridoma culture supernatants to influence lysosomal enzyme binding to purified MPR was assessed by a modification to the above procedure. After binding 5G5/17B5 antibodies to the wells, excess sheep anti-mouse Ig was blocked with 50 µl of an irrelevant mouse antibody (IgG_1) for 1 h. Wells were then incubated with 50 µl of the MPR preparation (2.5 µg/ml) for 2 h, followed by 50 µl of the test hybridoma supernatant for 30 min. Without removing the supernatant, 50 µl of lysosomal enzyme-enriched medium was added, and the plate incubated overnight at 4°C. Wells were washed, then incubated with β-glucuronidase substrate solution for 8 h at 37°C. β-glucuronidase activity was measured as before and the ability of the antibodies to influence lysosomal enzyme binding was compared with the effects of M6P and mannose.

3.2.11.2 The effect of antibodies on binding of fluorescent PPME to Clone 9 hepatocytes

Adherent Clone 9 hepatocytes were detached using 0.1% EDTA in PBS and resuspended in 10% FCS/RPMI medium at a density of $1x10^7$ cells/ml. Aliquots (20 µl) were placed in the wells of a V-bottomed 96-well plate ($2x10^5$ cells/well), and incubated with 20 µl of hybridoma culture supernatant for 30 min at 4°C. 40 µl of fl-PPME, diluted to 0.1 mg/ml in 10% FCS/RPMI medium (pH 7.0), was incubated with the cells for 30 min at 4°C. The cells were washed twice with cold 10% FCS/RPMI and once with cold PBS, and were fixed with 1% paraformaldehyde/PBS, pH 7.4. Fluorescence was measured by flow cytometry either immediately or the following day (Section 2.2.5). Median FIU values of mAb-treated samples were compared with those of cells allowed to bind PPME-FITC (i) in the absence of antibody and (ii) in the presence of 5 mM M6P. All samples were done in duplicate.

Results were corrected for autofluorescence, and the M6P- and antibody-treated samples expressed as a percentage of fl-PPME binding in the absence of antibody. Replicates from all experiments were averaged and SEM values calculated. Means of antibody-treated samples were compared with control fl-

PPME binding by Student's t test (Swinscow, 1978). p values less than 0.05 were considered to be statistically significant.

3.2.11.3 The effect of antibodies on uptake of fluorescent PPME by Clone 9 hepatocytes

Clone 9 hepatocytes were aliquoted into V-bottomed 96-well plates as described in Section 3.2.11.2, and incubated with 60 µl of hybridoma culture supernatant for 30 min at 4°C. 20 µl of fl-PPME diluted in 10% FCS/RPMI, pH 7.0 (0.25 mg/ml) was added and the cells incubated for a further 3 h at 37°C. Cells were washed twice with ice cold 10% FCS/RPMI medium and once with cold PBS, and fixed with 1% paraformaldehyde/PBS, pH 7.4. Fluorescence was measured either immediately or the following day by flow cytometry (Section 2.2.5) and median FIU values analysed as in Section 3.2.11.2.

3.2.11.4 The effects of PMP and PPME on binding of antibodies to Clone 9 hepatocytes

Clone 9 cells were aliquoted into V-bottomed microtitre plates as described in Section 3.2.11.2. 20 µl of PMP or PPME, diluted to 0.1 mg/ml in 10% FCS/RPMI (pH 7.0) was added to half the wells, and medium alone to the remaining wells, and the cells incubated for 1 h at 4°C. Each hybridoma supernatant (20 µl) was then added to (i) PMP/PPME-treated cells and (ii) control cells, and incubated for 30 min at 4°C. Cells were washed 3 times with cold 10% FCS/RPMI, then incubated for 30 min at 4°C with a saturating concentration of sheep anti-mouse Ig $F(ab')_2$ fragment, directly conjugated with fluorescein (Silenus, Hawthorne, Australia). Cells were washed twice in cold 10% FCS/RPMI medium and once in cold PBS, and fixed with 1% paraformaldehyde/PBS, pH 7.4. Fluorescence was measured either immediately or the following day by flow cytometry (Section 2.2.5). Median FIU values were corrected by subtracting the fluorescence due to binding of an irrelevant mouse IgG_1, specific for human endothelial cell antigens.

Antibody binding to PMP/PPME-treated samples were expressed as a percentage of binding to control cells. Replicates from all experiments were averaged and SEM values calculated. For each antibody, the mean binding to polysaccharide-treated cells was compared with the control value of 100% using Student's t test (Swinscow, 1978). p values <0.05 were considered statistically significant.

3.2.11.5 Ability of antibodies to deplete MPR-300 from the cell surface

U937 or Clone 9 cells were aliquoted (20 µl) into V-bottomed microtitre plates as described in Section 3.2.11.2. U937 cells were incubated for 2 h at 37°C with 20 µl of a rabbit antiserum specific for human MPR-300, with an irrelevant control antibody (specific for chicken histidine-rich glycoprotein), or with medium. Clone 9 cells were incubated with 80 µl of a mAb specific for rat MPR-300 (purified mAb 1G7/9H4 at 10 µg/ml, or one of several hybridoma culture supernatants), with isotype control antibodies (purified mAb specific for a murine erythrocyte enzyme at 10 µg/ml, or irrelevant hybridoma supernatant), or with medium. After incubation with antibody at 37°C, expression of cell surface MPR-300 on antibody-treated cells was measured by (a) immunofluorescent flow cytometry, or (b) uptake of fl-PPME, and compared with antibody binding or PPME uptake by untreated cells.

(a) Cell surface MPR-300 was measured by antibody binding at 4°C. Cells were washed once in cold 10% FCS/RPMI medium, then incubated for 30 min at 4°C with the same anti-MPR-300 antibody as in the previous incubation; cells treated with isotype control antibodies were also incubated with an anti-MPR-300 antibody. Each antibody was also used to stain MPR-300 on untreated control cells. Cells were washed three times with 10% FCS/RPMI, then incubated for 30 min at 4°C with a saturating concentration of a fluorescein-Protein A conjugate (Pharmacia, Uppsala, Sweden) in the case of U937 cells, or a fluoresceinated sheep anti-mouse Ig $F(ab')_2$ fragment (Silenus, Hawthorne, Australia) in the case of Clone 9 cells. Cells were then washed twice with 10% FCS/RPMI and once with PBS, fixed with 1% paraformaldehyde/PBS, pH 7.4, and analysed the following day by flow cytometry (Section 2.2.5). Fluorescence histograms of cells treated with anti-MPR-300 or isotype control antibodies were compared to those of the untreated control cells.

(b) MPR-mediated internalization of fl-PPME was also measured following incubation of cells with anti-MPR-300 or isotype control antibodies at 37°C. Cells were washed once with 10% FCS/RPMI medium, and 20 µl of fl-PPME (150 µg/ml in 10% FCS/RPMI, pH 7.0) added. Cells were incubated for 2 h at 37°C, washed and analysed by flow cytometry.

3.2.11.6 Effect of antibodies on IGF-II binding to MPR

3.2.11.6.1 IGF-II binding to purified MPR

Round-bottomed PVC microtitre plates (Dynatech Laboratories, Alexandria, VA) were coated overnight at 4°C with 100 µl of the MPR preparation diluted in coating buffer (2.5 µg/ml). Control wells were incubated with coating buffer only. Plates were blocked with 3% BSA/PBST for 2 h at 37°C, and washed with PBST. Serial dilutions of iodinated IGF-II (0-1 nM; specific activity = 2000 Ci/mol) were prepared in 1% BSA/PBST, and incubated with both MPR-coated and control wells (50 ml/well) for 3h at RT. Unbound ^{125}I-IGF-II was removed and the plate washed 5 times with PBST. Wells were removed from the plate with a hot wire and counted by an Auto-gamma 5650 counter (Packard Instrument International, Meriden, CT). Specific binding was determined by subtracting the binding of I^{125}-IGF-II to uncoated control wells from binding to MPR-coated wells.

3.2.11.6.2 Effect of antibodies on IGF-II binding to MPR

Round-bottomed PVC microtitre plates were coated overnight at 4°C with 100 µl of MPR preparation (2.5 µg/ml) diluted in coating buffer, or with coating buffer alone. Duplicate MPR-coated wells were included. All wells were blocked with 3% BSA/PBST for 2 h at 37°C, then incubated with 100 µl of hybridoma supernatant at a saturating concentration for 2 h at RT. Control wells were incubated with HT medium. Iodinated IGF-II (2 nM stock) was diluted to 1 nM with 1% BSA/PBST and 50 µl incubated with each well for 3 h at RT. Unbound ^{125}I-IGF-II was removed and the plate washed 5 times with PBST. Wells were removed from the plate with a hot wire and counted by an Auto-gamma 5650 counter. Specific binding was determined by subtracting the binding of I^{125}-IGF-II to uncoated wells from the mean binding to MPR coated wells. Binding to each MPR-antibody complex was expressed as a percentage of binding to MPR-coated wells in the absence of antibody.

3.2.12 The effect of a purified mAb specific for MPR-300 on passively-induced EAE

Single cell suspensions were prepared from the spleens of Lewis (JC) rats sensitized ten days previously with 25 µg MBP emulsified in Freund's Complete Adjuvant. Cells were cultured at a density of 2x10^6 cells/ml in 10% FCS/RPMI 1640 medium, and stimulated with 2 µg/ml Con A for 72 h at 37°C. Cells were harvested by centrifugation, washed three times in Hank's Balanced

Salt Solution and resuspended in serum-free RPMI 1640 at a density of 10^7 cells/ml. One third of the cells were incubated with purified 1G7/9H4 antibody (100 µg/ml) for 45 min at 37°C, and the remaining cells with an isotype control antibody (specific for a murine erythrocyte enzyme). Cells were washed twice with serum-free RPMI, resuspended in RPMI at a concentration of $3x10^7$ cells/ml and injected i.v. into three groups of 4-6 healthy recipients (1 ml/rat). Groups A and C received cells preincubated with the isotype control mAb, while group B received cells preincubated with 1G7/9H4 mAb. Rats were injected i.p. with antibody at the time of cell transfer, and on days 1, 2, 3, 4 and 6 at a dosage of 6 mg/kg. Group A received the isotype control mAb, while groups B and C received the 1G7/9H4 mAb.

Rats were examined daily for clinical signs of EAE and scored on an arbitrary scale of severity from 0 to 5 where: 0 = asymptomatic, 1 = distal half of tail flaccid, 2 = entire tail flaccid, 3 = ataxia, difficulty in righting reflex, 4 = hindlimb weakness, 5 = hindlimb paralysis. Mean clinical scores were calculated for each day of treatment, and groups B and C compared with group A using Student's t test (Swinscow, 1978) and Spearman's Rank test (Moroney, 1951).

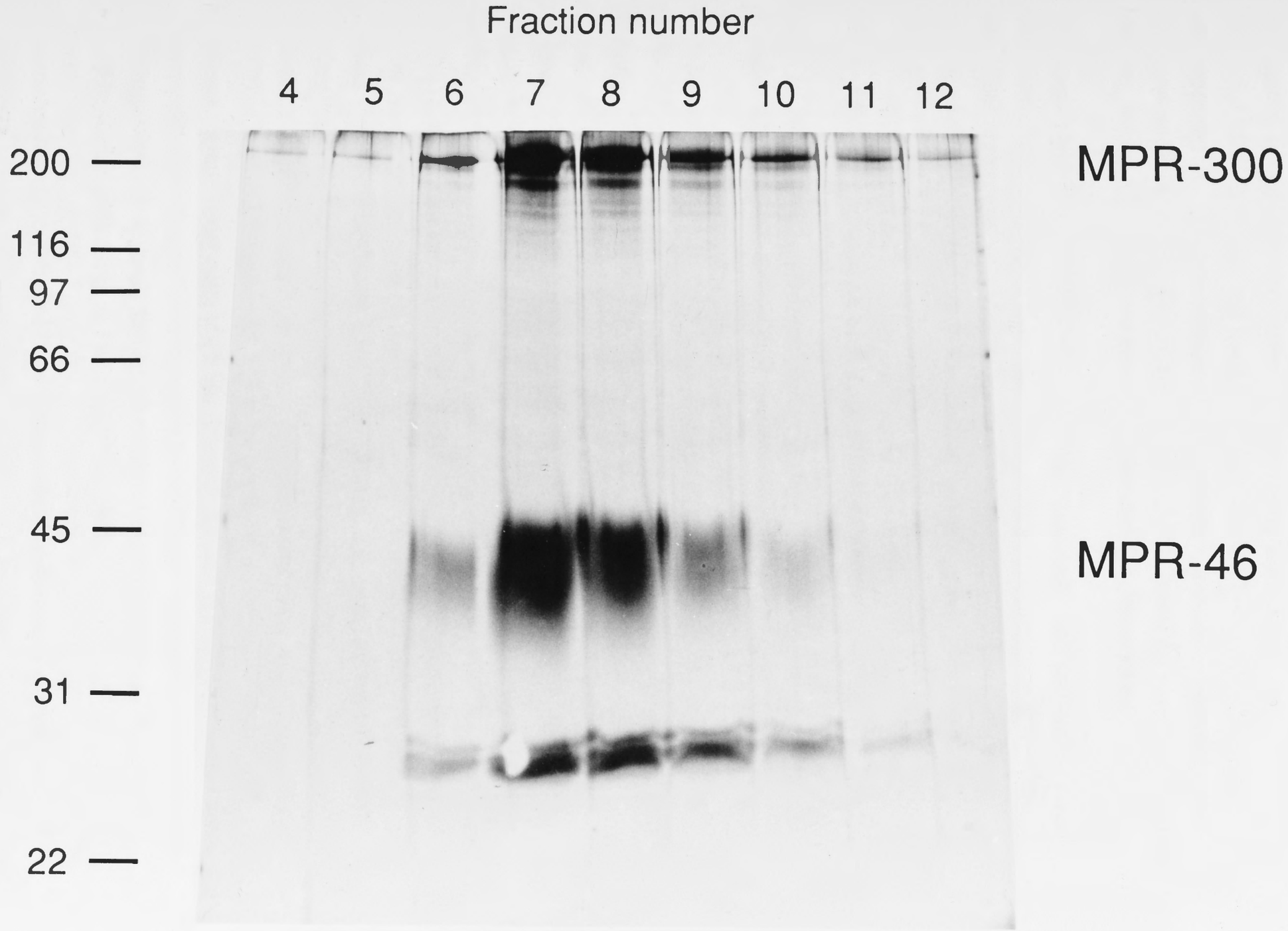
3.3 RESULTS

3.3.1. MPR purification

Rat liver was selected as the source of MPR as earlier experiments, demonstrating the inhibitory effect of M6P on inflammation in EAE and adjuvant arthritis, were performed in rats, and it was anticipated that any biologically active mAbs would be tested in the EAE model. A mixture of MPR-300 and MPR-46 was purified from rat liver by affinity chromatography on PMP-Sepharose 4B. A typical elution pattern, as assessed by SDS-PAGE, is shown in Fig. 3.1. The two MPRs were eluted either competitively with M6P or with a low pH buffer, the latter taking advantage of the decrease in binding affinity for M6P-bearing ligands below pH 6 (Hoflack *et al.*, 1987; Tong and Kornfeld, 1989). Both the low pH sodium acetate buffer and M6P were able to elute all M6P-binding proteins from the column. MPR-46 eluted more readily than MPR-300, usually being released over a 4-5 ml volume. The elution volume for MPR-46 was similar regardless of the buffer used, thus both were equally suitable for MPR-46 elution. The bulk of MPR-300 eluted with MPR-46, although small amounts continued to elute slowly from the column with additional washing. This "tail" of MPR-300 was released more rapidly by low

Fig. 3.1

Analysis of M6P-binding proteins purified from rat liver by affinity chromatography on PMP-Sepharose 4B. Bound protein was eluted with sodium acetate buffer (pH 4.5). Samples (30 µl) from the column fractions indicated were electrophoresed on a 10% SDS-polyacrylamide gel under reducing conditions. Protein was stained with silver nitrate. Molecular weight standards are indicated in kDa.


Fraction number
4
5
6
7
8
9
10
11
12
200
116
97
66
45
31
22
MPR-300
MPR-46

pH than by M6P. Sodium acetate buffer was therefore a more efficient elution buffer for MPR-300, and was used more frequently. Two additional proteins, with apparent M_r of approximately 27 kDa, were consistently eluted by both M6P and sodium acetate elution buffers (Fig. 3.1), but not by mannose (data not shown), suggesting that these proteins were also specific for M6P.

Although MPR-300 and MPR-46 can be separated from one another by size-exclusion chromatography (Li *et al.*, 1989), mice were immunized with the mixed MPR preparation. Multiple batches of the MPR preparation were pooled and concentrated, and used to immunize a group of seven BALB/c mice.

3.3.2. MAb production and characterization

3.3.2.1. Screening hybridoma supernatants by antibody capture on purified MPR

Two fusions were performed in an attempt to produce mAbs against both MPR-300 and MPR-46. Tissue culture plates from fusion 1 were scored for the presence of colonies on day 12 post fusion. Supernatants from colony-containing wells were collected at either day 13 or day 21 post-fusion and screened for antibody binding to components of the purified MPR preparation. In order to maximise the sensitivity of antibody detection, a time-resolved fluorescence assay (DELFIA) was used in preference to an ELISA. A negative control (preimmune mouse serum), and a positive control (immune mouse serum) was included in each assay. Hybridomas were considered to be producing specific antibody if the fluorescence, corrected for non-specific binding, exceeded 30 000 fluorescence intensity units (FIU). Fig 3.2 illustrates the results for 48 supernatants from a representative plate, collected on day 13. Of 1776 supernatants screened, 549 were positive, and the 305 giving the strongest signals were expanded into 24-well plates. These hybridomas were grown until the cultures filled between 2 and 12 wells, then were harvested by centrifugation and frozen. The spent medium from these hybridomas was stored in sterile containers at 4°C for further characterization.

Supernatants from expanded cultures were retested using the same assay. On comparison with controls (negative = 25, 000 FIU; positive = 700, 000 FIU), forty were considered to be weakly positive (40, 000 - 100, 000 FIU) and nine strongly positive (over 100 000 FIU). These nine hybridomas, designated 1G2, 4F5, 7B3, 9H4, 11G3, 13B7, 13C11, 21F10 and 22D3 on the basis of their position in the

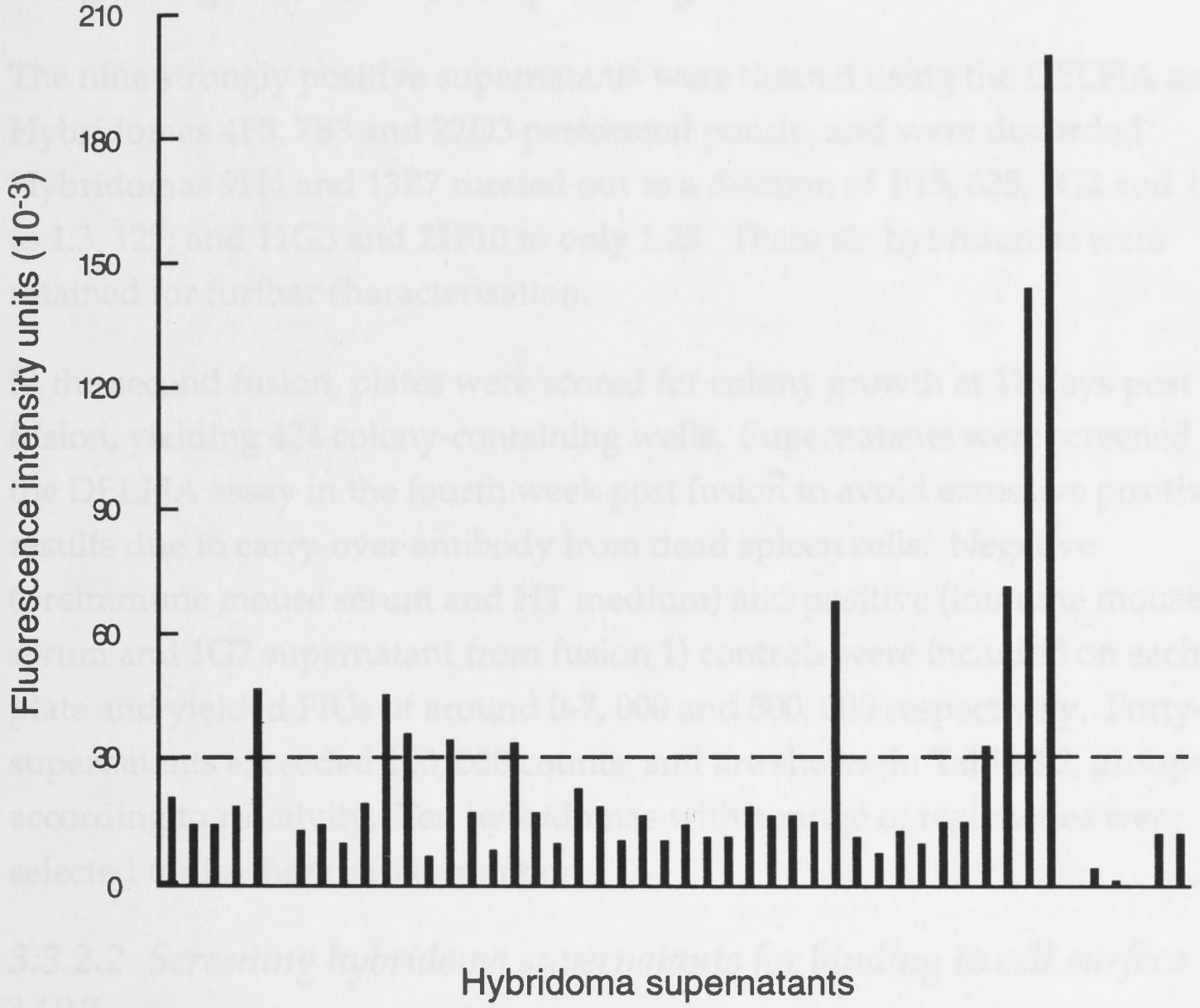


Fig. 3.2

Binding of fusion-1 hybridoma supernatants to an immobilized MPR preparation. Binding was assessed using the DELFIA assay described in Section 3.2.8.1. Non-specific binding was subtracted from all samples. A positive control (immune mouse serum) gave a value of 40, 000 FIU, and a negative control (preimmune mouse serum) was below zero. Supernatants giving > 30, 000 FIU (indicated by the dotted line) were considered to show significant specific binding.

original microtitre plates, were retained for further investigations. The remaining 256 supernatants were negative. This loss of reactivity after prolonged culture was probably due to detection of carry-over antibody produced by unfused spleen cells in the original screening assay, and/or to overgrowing of cultures by non-producing hybridomas.

The nine strongly positive supernatants were titrated using the DELFIA assay. Hybridomas 4F5, 7B3 and 22D3 performed poorly, and were discarded. Hybridomas 9H4 and 13B7 titrated out to a dilution of 1:15, 625; 1G2 and 13C11 to 1:3, 125; and 11G3 and 21F10 to only 1:25. These six hybridomas were retained for further characterisation.

In the second fusion, plates were scored for colony growth at 11 days post fusion, yielding 424 colony-containing wells. Supernatants were screened by the DELFIA assay in the fourth week post fusion to avoid excessive positive results due to carry-over antibody from dead spleen cells. Negative (preimmune mouse serum and HT medium) and positive (immune mouse serum and 1G2 supernatant from fusion 1) controls were included on each plate and yielded FIUs of around 0-7, 000 and 500, 000 respectively. Forty-eight supernatants exceeded 100, 000 counts, and are shown in Table 3.2, grouped according to reactivity. Ten hybridomas with a range of reactivities were selected for further characterization.

3.3.2.2 Screening hybridoma supernatants for binding to cell surface MPR

The sixteen hybridomas selected from the two fusions were screened for antibody binding to the cell surface of the rat Clone 9 hepatocytes by immunofluorecent flow cytometry. This identified antibodies able to bind to the extracellular portion of the MPRs in their native conformation. Clone 9 cells express both MPR-300 and MPR-46 on intracellular membranes (Matovcik *et al.*, 1990), and MPR-300 has been detected at the cell surface (Brown *et al.*, 1984). As MPR-300 and MPR-46 have a similar subcellular distribution in other cell types (Bleekemolen *et al.*, 1988), MPR-46 was assumed also to be present at the cell surface. Representative fluorescence histograms are shown in Fig. 3.3. Fig 3.4 illustrates that the supernatants from hybridomas 21F10, 2E3 and 6C4 bound weakly to Clone 9 cells, with fluorescence comparable to that of the negative control. These were omitted from further investigations, as was 18C10, whose binding was also low. The remaining supernatants showed

Table 3.2

Binding of hybridoma supernatants from fusion-2 to purified MPR preparation.

Fluorescence intensity units (x 10^{-3}) [a]					
100 - 199	200 - 299	300 - 399	400 - 499	500 - 599	> 600
1G1[b]	**2E3[c]**	1C11	**5G8[c]**	15C8	**5F3[c]**
1B4	**13C6[c]**	5C7	10D5	16B9	5F10
1C4	13C8	5G10	12F1		15F10
1A7	14C4	**6C4[c]**			**17B5[c]**
6C7	**15E4[c]**	7C2			17B6
7E6		8C5			
8E9		12F12			
9E5		16A3			
9A8		**18F4[c]**			
10E6					
10E8					
12E10					
15H3					
16G7					
17E9					
18C2					
18D5					
18C10[c]					
19A10					
20F4					
20F7[c]					
20D10					
20E11					

[a] Antibody binding to purified MPR, immobilized on PVC, was assessed using the DELFIA assay (Section 3.2.8.1). Background fluorescence (due to binding of an irrelevant mAb) was in the range 0 - 8000 FIU.

[b] Well number, based on position in original 96-well plates

[c] Hybridomas retained for further characterization

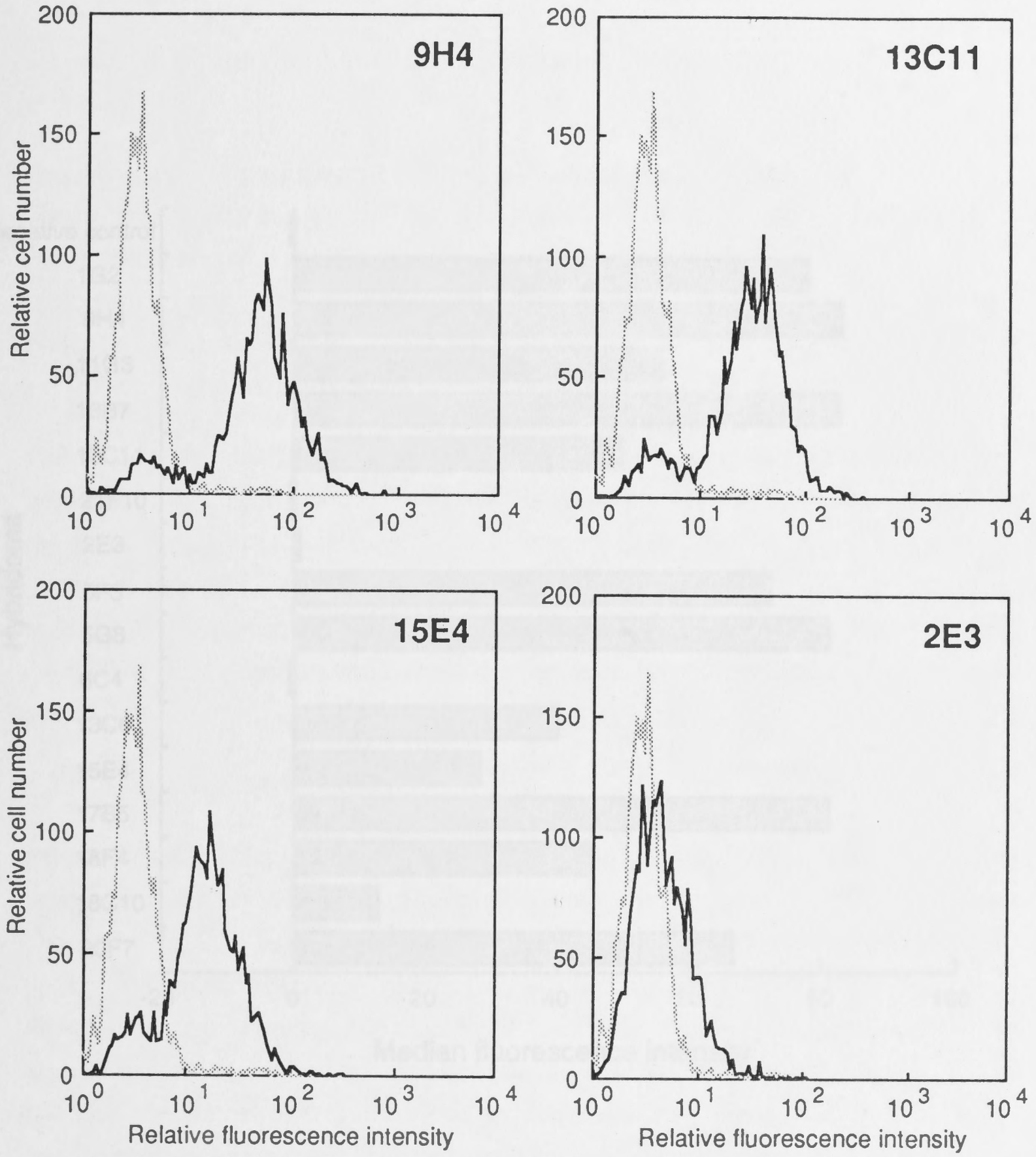


Fig. 3.3

Binding of hybridoma supernatants to cell surface MPR on Clone 9 hepatocytes, as assessed by immunofluorescent flow cytometry. Hybridoma supernatants (——) were compared with binding of a control mAb specific for human endothelial cell surface antigens (---).

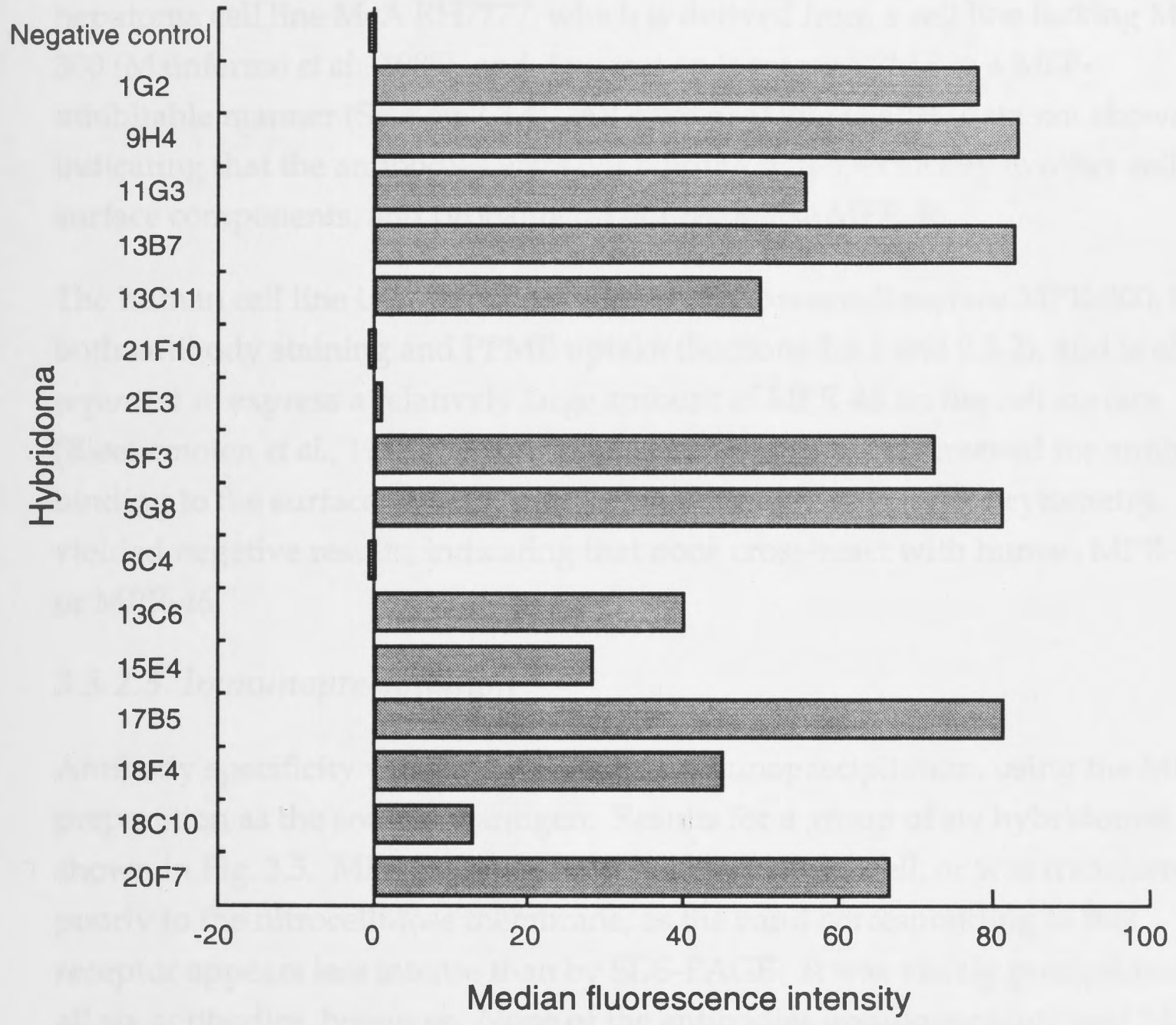


Fig. 3.4

Binding of hybridoma supernatants to Clone 9 hepatocyte cell surface MPR, as assessed by immunofluorescent flow cytometry. Values shown are the means of duplicate samples. Non-specific staining, due to binding of the fluoresceinated anti-mouse Ig reagent, was subtracted from all samples. Antibody binding was compared with that of a control mAb specific for human endothelial cell surface antigens.

variation in binding with the strongest being 1G2, 9H4, 13B7, 5F3, 5G8, 17B5 and 20F7.

The twelve positive supernatants were screened for binding to the rat hepatoma cell line McA RH7777, which is derived from a cell line lacking MPR-300 (Mainferme *et al.*, 1985), and does not endocytose PPME in a M6P-inhibitable manner (Section 2.3.4). All gave negative results (data not shown), indicating that the antibodies were not binding non-specifically to other cell surface components, and probably do not recognise MPR-46.

The human cell line U937 has been shown to express cell surface MPR-300, by both antibody staining and PPME uptake (Sections 2.3.1 and 2.3.2), and is also reported to express a relatively large amount of MPR-46 on the cell surface (Bleekemolen *et al.*, 1988). Hybridoma supernatants were screened for antibody binding to the surface of U937 cells by immunofluorescent flow cytometry. All yielded negative results, indicating that none cross-react with human MPR-300 or MPR-46.

3.3.2.3 Immunoprecipitation

Antibody specificity was determined by immunoprecipitation, using the MPR preparation as the source of antigen. Results for a group of six hybridomas are shown in Fig. 3.5. MPR-300 either did not biotinylate well, or was transferred poorly to the nitrocellulose membrane, as the band corresponding to this receptor appears less intense than by SDS-PAGE. It was visibly precipitated by all six antibodies, however. None of the antibodies immunoprecipitated MPR-46. The 27 kDa protein(s) described earlier bound non-specifically to control beads (preincubated with HT medium); antibodies were regarded as binding specifically to these proteins if the band was more intense than that of the control. Several contaminating bands, with molecular weights intermediate between MPR-300 and MPR-46, were evident in the biotinylated MPR preparation. These may be degradation products, as they were more apparent in older MPR preparations than in a freshly prepared one (data not shown). They bound non-specifically to control beads, and in greater amounts to some of the antibodies.

Results for all hybridomas are summarised in Table 3.3. All antibodies except 20G7 bound MPR-300; the reactivity of 20G7 was unclear. Antibodies produced by the hybridomas 9H4, 13C11, 5F3, 5G8, 13C6, 17B5 and 20F7 also bound the 27 kDa protein(s). None of the antibodies bound to MPR-46.

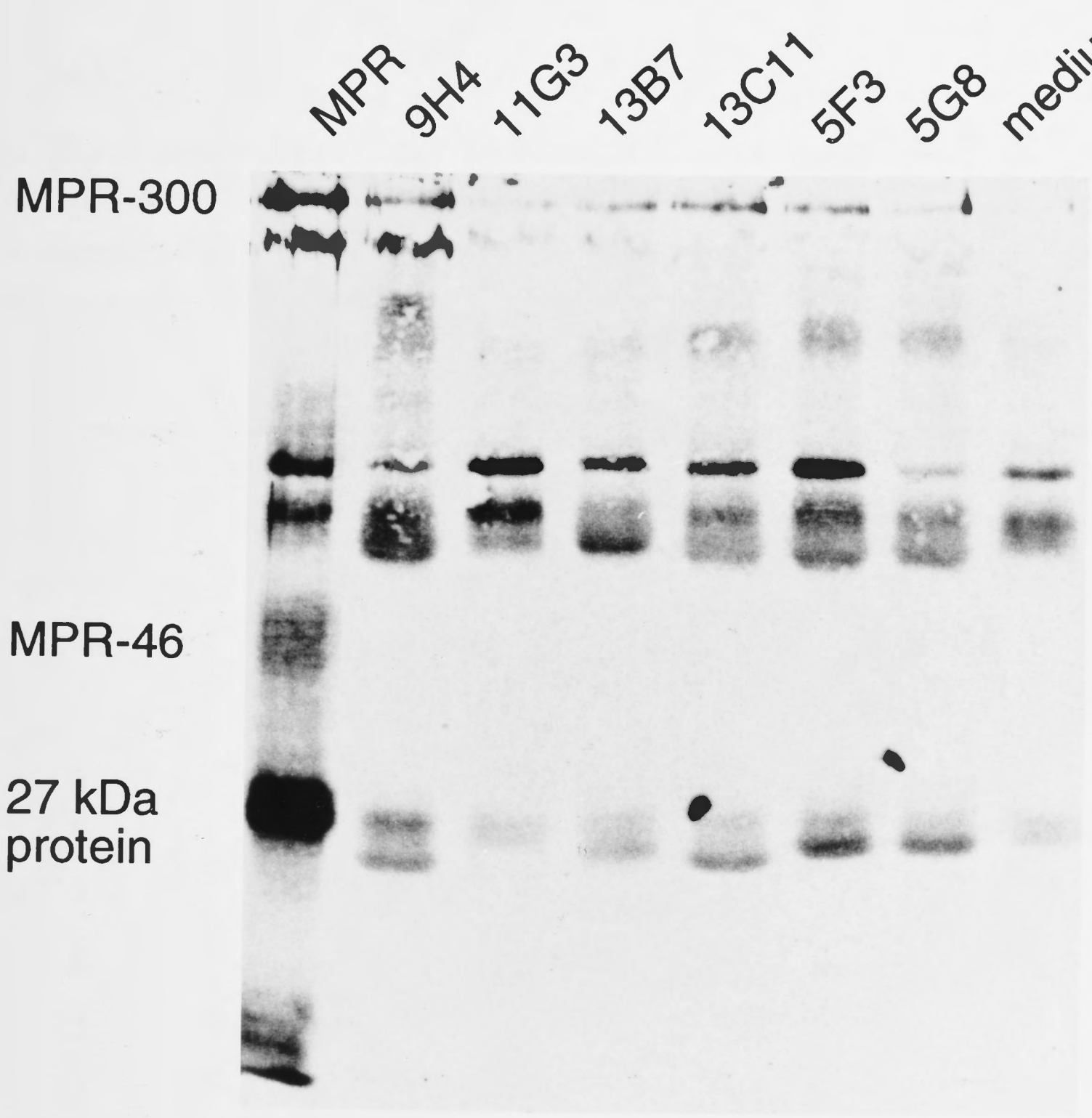


Fig. 3.5

Analysis of hybridoma supernatants by immunoprecipitation. Antibodies were adsorbed onto Protein G-Sepharose, and the beads incubated with a biotinylated MPR preparation. Bound protein was released by boiling in SDS sample buffer, separated by SDS-PAGE, and electroblotted onto a nitrocellulose membrane. Biotinylated proteins were detected by probing the membrane with streptavidin-HRP complex, and visualized using an ECL Western blot detection reagent (Amersham).

Table 3.3

Ability of antibodies to immunoprecipitate biotinylated MPR-300, MPR-46 and an associated 27 kDa protein.

Fusion no.	Hybridoma	Reactivity with MPR preparation		
		MPR-300	MPR-46	27 kDa protein
1	1G2	+	–	–
1	9H4	+	–	+
1	11G3	+	–	–
1	13B7	+	–	?
1	13C11	+	–	+
2	5F3	+	–	+
2	5G8	+	–	+
2	13C6	+	–	+
2	15E4	–	–	–
2	17B5	+	–	+
2	18F4	+	–	–
2	20F7	?	–	+

+ antibody reacts with MPR component
– antibody does not react with MPR component
? reactivity unclear

Supernatant from 15E4 failed to bind any of the bands of interest; this hybridoma was subsequently shown to secrete IgM antibodies, hence this mAb was unable to bind to the Protein G beads.

3.3.3 Cloning of hybridomas

3.3.3.1 Selection of hybridomas for cloning

Hybridoma supernatants were allocated to one of four groups binding different epitopes on MPR-300 on the basis of additive binding to Clone 9 cells. The hybridoma 17B5 was cloned by limiting dilution, and one clone (5G5/17B5) chosen as the initial reference antibody. A saturating amount of 5G5/17B5 (determined by titration) and a second, uncloned, hybridoma supernatant were incubated simultaneously with Clone 9 hepatocytes, and the bound antibodies detected by immunofluorescent flow cytometry. Fluorescence was compared with that resulting from binding of the two antibodies individually. Where the second antibody bound to the same epitope as 5G5/17B5, its presence did not increase the fluorescence over that produced by the first antibody alone. If, however, the second antibody bound to a different epitope, then the resulting fluorescence was close to the sum of that produced by the two antibodies individually. Eleven supernatants were sorted into two groups on this basis, those that bound to the same epitope as 5G5/17B5 (non-additive), and those that did not (additive) (Fig. 3.6). The process was then repeated on the additive group, selecting a new reference antibody, until all supernatants were allocated to one of four groups. The results are summarized in Table 3.4.
Representatives from each group were then selected for cloning.

3.3.3.2 Cloning of hybridomas

The seven hybridomas 5G8 and 17B5 (group 1), 9H4 (group 2), 13C11 and 13C6 (group 3), and 15E4 and 20F7 (group 4) were cloned by limiting dilution. 5G8, 17B5 and 15E4 were cloned from growing cultures, while 9H4, 13C11, 13C6 and 20F7 were thawed from frozen stocks and cultured for 14 days before cloning. Supernatants from wells identified as containing single colonies were assayed for binding to the purified MPR preparation by the DELFIA assay. Positive supernatants were assayed for binding to cell surface MPR on Clone 9 hepatocytes. All seven hybridomas produced between 17 and 67 colonies that gave positive results in both assays. Results are summarised in Table 3.5. All positive clones were expanded to fill at least 2 wells of a 24-well tissue culture plate, and were frozen for storage. From each hybridoma, six clones which

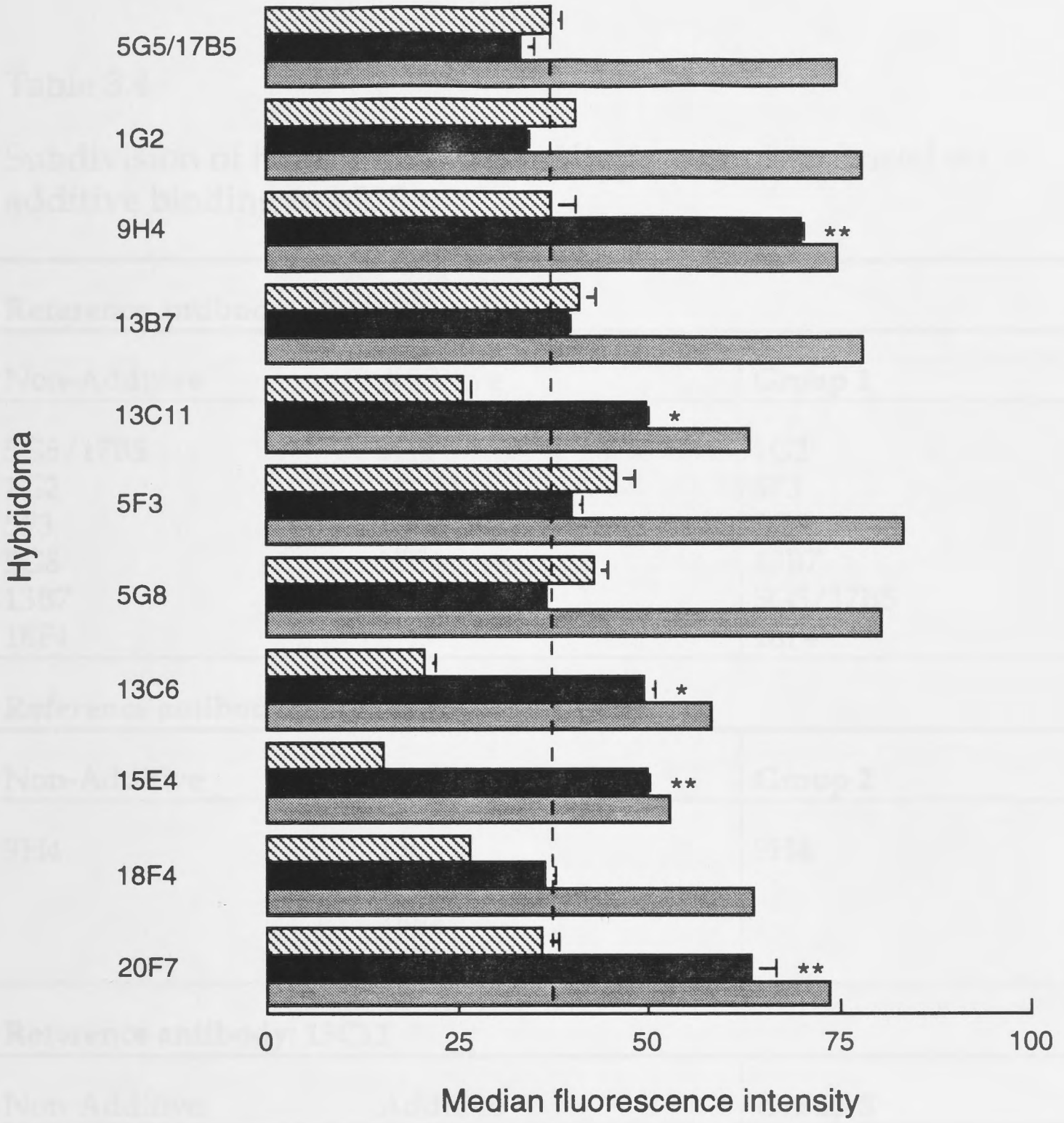


Fig. 3.6

Additive binding of hybridoma supernatants to Clone 9 hepatocytes. A saturating amount of mAb 5G5/17B5 was incubated with cells at 4°C, together with a second, uncloned hybridoma supernatant. Bound antibodies were detected by immunofluorescent flow cytometry. Each value is the mean ± SEM where n=3 (one experiment in triplicate). The dotted line represents fluorescence due to mAb 5G5/17B5. Data is expressed as fluorescence due to hybridoma supernatant alone (▨), or in combination with mAb 5G5/17B5 (■), with the theoretical maximum fluorescence if the two antibodies have completely different binding sites depicted as ▩ . A statistically significant increase over 5G5/17B5 binding is indicated by * ($p<0.05$) and ** ($p<0.02$).

Table 3.4

Subdivision of hybridomas into epitope groupings based on additive binding to MPR[a].

Reference antibody: 5G5/17B5		
Non-Additive	Additive	**Group 1**
5G5/17B5	9H4	1G2
1G2	13C11	5F3
5F3	13C6	5G8
5G8	15E4	13B7
13B7	20F7	5G5/17B5
18F4		18F4

Reference antibody: 9H4		
Non-Additive	Additive	**Group 2**
9H4	13C11	9H4
	13C6	
	15E4	
	20F7	

Reference antibody: 13C11		
Non-Additive	Additive	**Group 3**
13C11	15E4	13C6
13C6	20F7	13C11
		Group 4
		15E4
		20F7

[a] Hybridomas were allocated to one of four groups on the basis of their ability to bind to MPR on Clone 9 hepatocytes simultaneously with mAbs 5G5/17B5, 9H4 or 13C11, as assessed by immunofluorescent flow cytometry.

Table 3.5

Cloning of hybridomas

Hybridoma	Epitope group	Frequency of clones	Binding of mAbs to purified MPR preparation	Binding of mAbs to Clone 9 cell surface
5G8	1	77/470 = 16%	50/51	50/50
17B5	1	89/432 = 21%	67/71	67/67
9H4	2	40/384 = 10%	40/40	40/40
13C6	3	58/384 = 15%	46/49	46/46
13C11	3	64/384 = 17%	27/48	27/27
15E4	4	63/476 = 13%	17/30	17/17
20F7	4	63/384 = 16%	52/57	52/52

bound strongly to Clone 9 cells were expanded to fill a 24-well plate, the cells frozen and the medium stored in sterile containers at 4°C for future use.

3.3.3.3 Immunoglobulin subclass of monoclonal antibodies

The antibody isotype of six to twelve clones of each of hybridoma was determined by ELISA. All 15E4 clones produced IgM antibodies, while all clones of the remaining six hybridomas produced IgG_1.

3.3.4 Functional activity of antibodies

3.3.4.1 The effect of antibodies on ligand binding to the M6P binding site

3.3.4.1.1 Lysosomal enzyme binding to purified MPR

All twelve antibodies (both cloned and uncloned) were tested for their ability to inhibit the MPR-ligand interaction. This required a sensitive assay for ligand binding, using a suitable ligand. As lysosomal enzymes are the most biologically relevant ligand, an assay was developed to measure binding of lysosomal enzymes to purified MPR. Spent medium from Clone 9 hepatocytes cultured in the presence of 10 mM ammonium chloride was used as the source of lysosomal enzymes. Weak bases such as ammonium chloride raise the pH of acidic organelles and so prevent release of lysosomal enzymes into the prelysosomal compartment. Thus, the cell becomes depleted of unoccupied receptors, and newly synthesized lysosomal enzymes are secreted via the secretory pathway with M6P markers intact (von Figura and Hasilik, 1986). The lysosomal enzyme-enriched medium was incubated with purified MPR immobilised on PVC plates, and binding of β–glucuronidase detected using the derivatised substrate 4-methylumbelliferyl-β-D-glucuronide, which the enzyme cleaves to yield the fluorescent product 4-methylumbelliferone. Fig. 3.7 shows that production of the fluorescent product was dependent on the presence of both MPR and lysosomal enzymes, and was inhibited by M6P but not by mannose.

When antibodies were allowed to bind to immobilised MPR before adding the lysosomal enzyme-enriched medium, lysosomal enzyme binding was enhanced rather than inhibited, in some cases by 10-fold (data not shown). Such strong enhancement seemed an unlikely result, especially in view of the effects of the antibodies on PPME binding to cell surface MPR (described below). A more likely explanation is that the antibodies cross-linked and stabilized the immobilized MPR on the plate, so that ligand binding was

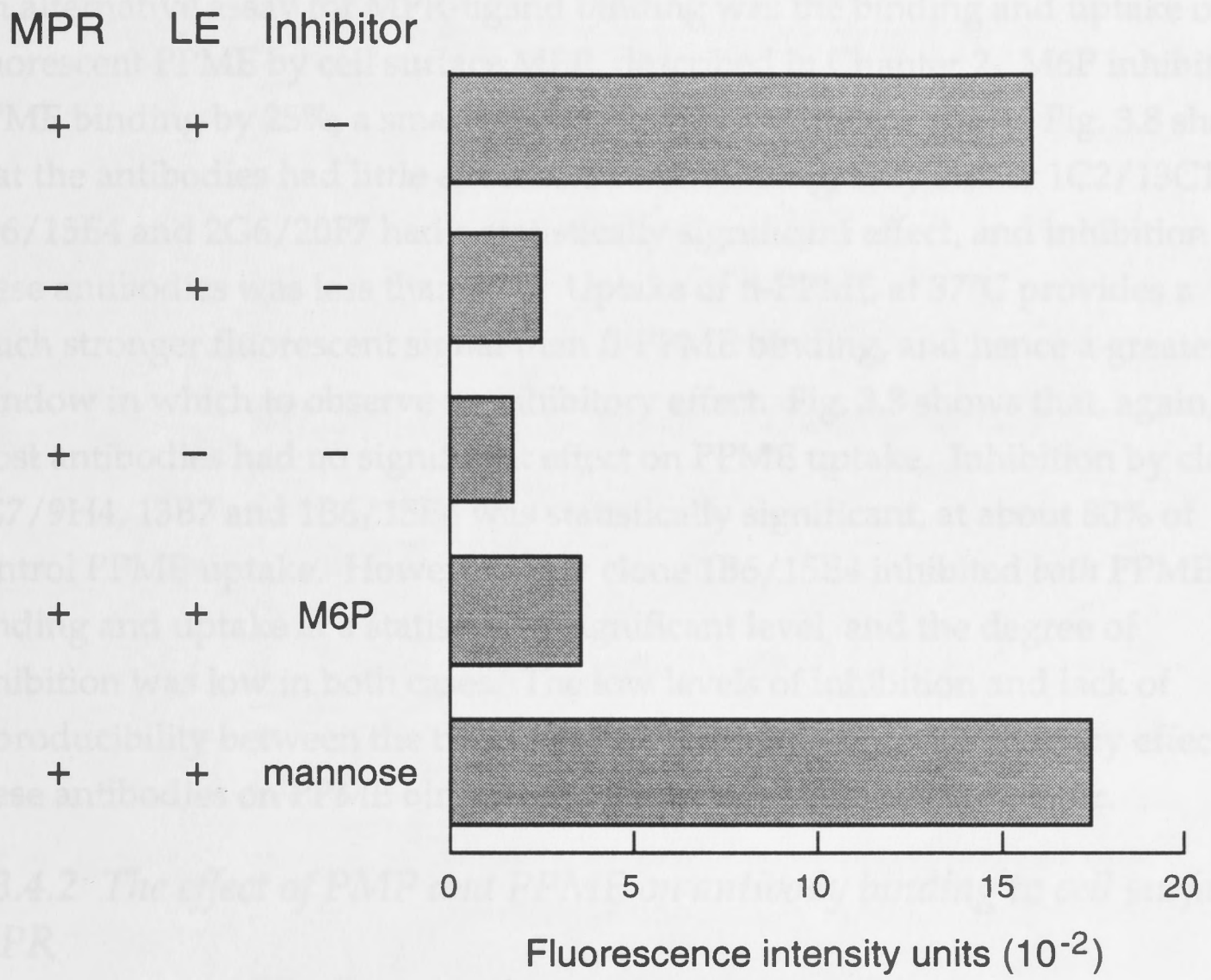


Fig. 3.7

Binding of lysosomal enzymes (LE) to purified MPR immobilized on a PVC plate via anti-MPR mAb (5G5/17B5). Enzyme binding was assessed by measuring bound β-glucuronidase activity. Inhibitors included in the assay were M6P and mannose, both at 10 mM.

greater than when the test antibody was not included. This technique, while suitable for measuring lysosomal enzyme binding to MPR, was clearly not suitable for detecting the inhibition of such binding by mAbs.

3.3.4.1.2 Binding and uptake of fl-PPME by Clone 9 hepatocytes

An alternative assay for MPR-ligand binding was the binding and uptake of fluorescent PPME by cell surface MPR, described in Chapter 2. M6P inhibited PPME binding by 25%, a small but statistically significant effect. Fig. 3.8 shows that the antibodies had little effect on PPME binding; only clones 1C2/13C11, 1B6/15E4 and 2G6/20F7 had a statistically significant effect, and inhibition by these antibodies was less than 10%. Uptake of fl-PPME at 37°C provides a much stronger fluorescent signal than fl-PPME binding, and hence a greater window in which to observe an inhibitory effect. Fig. 3.8 shows that, again, most antibodies had no significant effect on PPME uptake. Inhibition by clones 1G7/9H4, 13B7 and 1B6/15E4 was statistically significant, at about 80% of control PPME uptake. However, only clone 1B6/15E4 inhibited *both* PPME binding and uptake at a statistically significant level, and the degree of inhibition was low in both cases. The low levels of inhibition and lack of reproducibility between the two sets of experiments suggests that any effects of these antibodies on PPME binding to cell surface MPR-300 are minor.

3.3.4.2 The effect of PMP and PPME on antibody binding to cell surface MPR

The effects of PMP and PPME on binding of antibodies to cell surface MPR were assessed, since antibody binding to a site that includes the M6P binding site might be blocked by prior binding of ligand. PPME was expected to be more effective, as it is larger than PMP (approximately 2000 kDa compared to 1 kDa) and so should obscure a greater area around the ligand binding site. PMP, on the other hand, would only interfere with antibodies that bind very close to the M6P binding site. Fig. 3.9 shows that again, only a few antibodies were significantly affected by the presence of either ligand. PMP inhibited the binding of clones 1G7/9H4, 13B7, 1C2/13C11 and 5E1/5G8 by 20-40%. PPME inhibited binding of clones 1C2/13C11, 5F3, 5G8, 5G5/17B5 and 2G6/20F7 by 30-50%. Binding of only two antibodies, clone 1C2/13C11 and 5G8, was inhibited by both PMP and PPME, suggestive of antibody binding in the vicinity of the binding site, but these antibodies did not themselves inhibit ligand binding by more than 10% (Fig. 3.8). In general, the lack of reproducibility between experiments, and the relatively small effects observed,

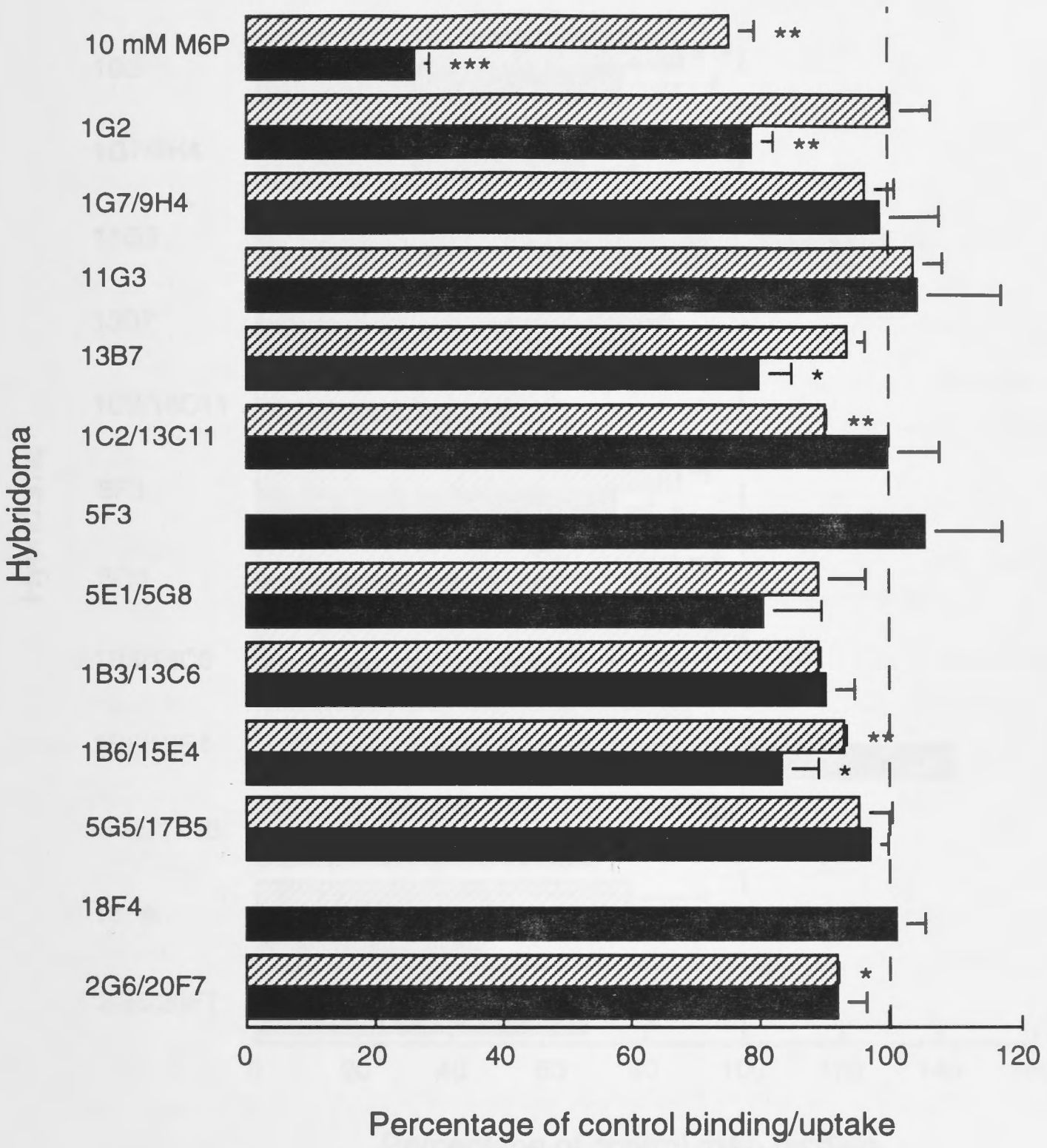


Fig. 3.8

Inhibition of fluorescent PPME binding (■) and uptake (▨) to Clone 9 hepatocytes by anti-MPR-300 mAbs , as measured by fluorescent flow cytometry. Each value is the mean ± SEM where n=4 for PPME binding and n=6 for PPME uptake. The dotted line represents control binding/uptake, i.e.100%. Statistically significant changes from 100% are indicated by * ($p<0.05$), ** ($p< 0.01$) and *** ($p<0.001$).

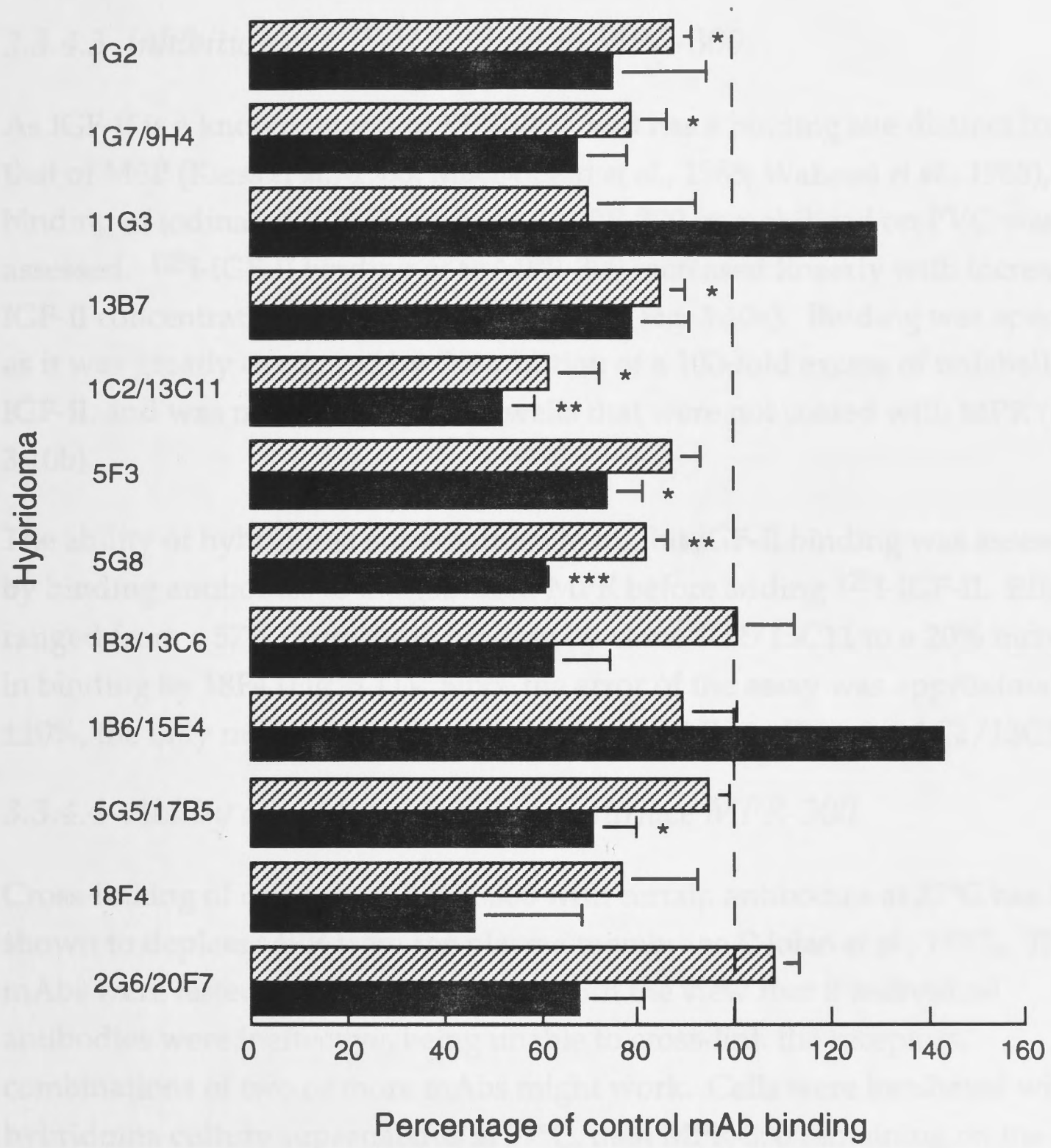


Fig. 3.9

Inhibition of anti-MPR mAb binding to Clone 9 hepatocytes by PMP (▨) and PPME (■), as measured by immunofluorescent flow cytometry. Each value is the mean ± SEM where n=4 for PMP inhibition and n=6 or 9 for PPME inhibition. The dotted line represents mAb binding in the absence of PMP or PPME (100%). Statistically significant changes from 100% are indicated by * ($p<0.05$), ** ($p< 0.01$) and *** ($p<0.001$).

again suggest that neither PMP or PPME binding interferes with the antibody binding sites.

3.3.4.3 Inhibition of IGF-II binding to MPR-300

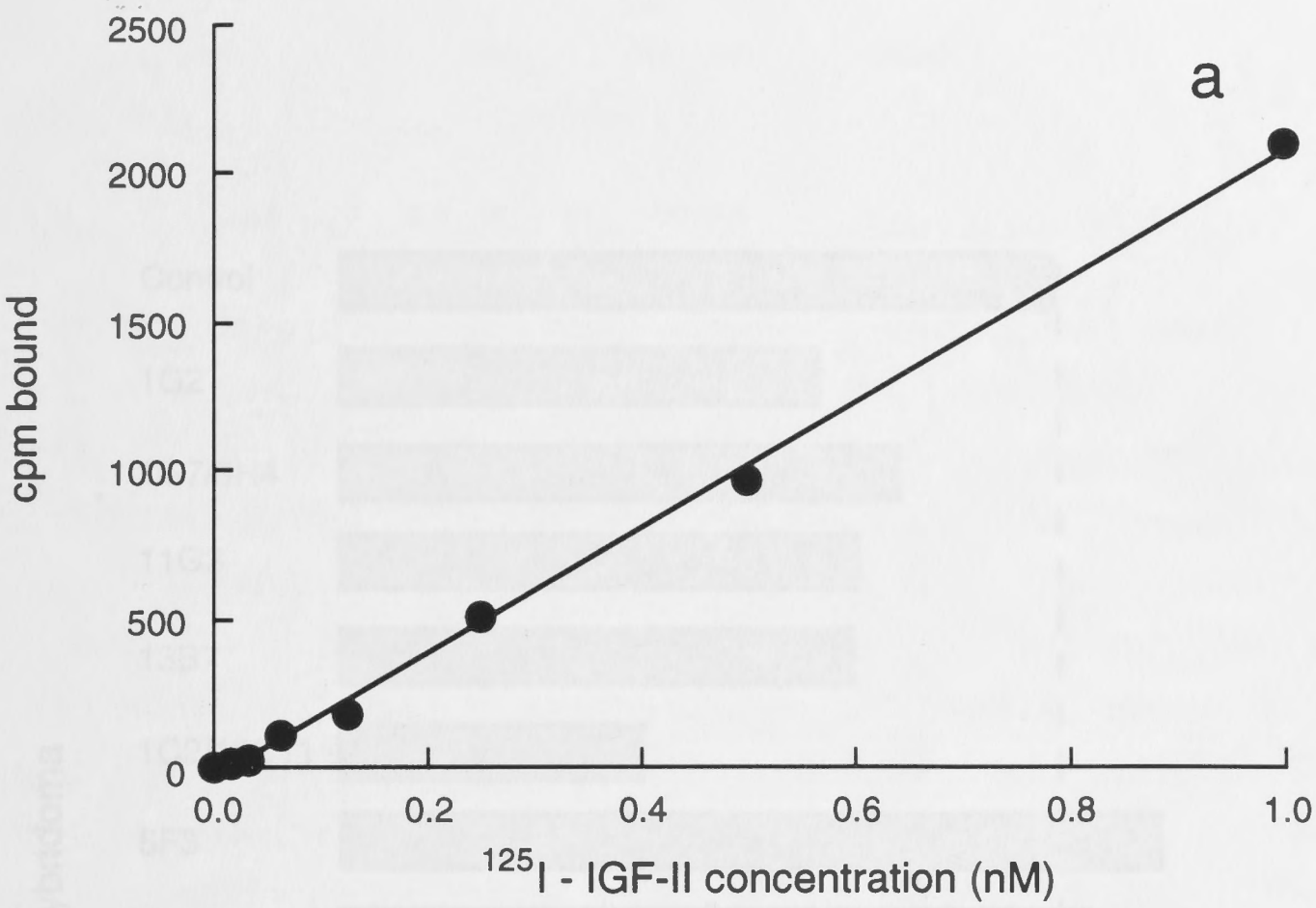
As IGF-II is a known ligand of MPR-300, and has a binding site distinct from that of M6P (Kiess *et al.*, 1988; MacDonald *et al.*, 1988; Waheed *et al.*, 1988), binding of iodinated IGF-II to purified MPR-300 immobilized on PVC was assessed. ^{125}I-IGF-II binding of to MPR-300 increased linearly with increasing IGF-II concentration, over the range 0-1 nM (Fig. 3.10a). Binding was specific, as it was greatly decreased by the addition of a 100-fold excess of unlabelled IGF-II, and was minimal in control wells that were not coated with MPR (Fig. 3.10b).

The ability of hybridoma supernatants to inhibit IGF-II binding was assessed by binding antibodies to immobilized MPR before adding ^{125}I-IGF-II. Effects ranged from a 57% decrease in binding by clone 1C2/13C11 to a 20% increase in binding by 18F4 (Fig. 3.11). Since the error of the assay was approximately ±10%, the only mAb which clearly inhibited IGF-II binding was 1C2/13C11.

3.3.4.4 Ability of mAbs to deplete cell surface MPR-300

Cross-linking of cell surface MPR-300 with certain antibodies at 37°C has been shown to deplete MPR from the plasma membrane (Nolan *et al.*, 1987). The mAbs were tested for a similar ability, with the view that if individual antibodies were ineffective, being unable to cross-link the receptors, combinations of two or more mAbs might work. Cells were incubated with hybridoma culture supernatants at 37°C, then MPR-300 remaining on the cell surface was detected by both immunofluorescent flow cytometry, using the same mAb as a probe, and by the ability of cells to endocytose fluorescent PPME. Cell surface MPR-300 was compared with MPR-300 expression by untreated, control cells.

Initially, the rabbit antiserum specific for human MPR-300 was tested on U937 cells, but did not noticably affect cell surface expression (data not shown). Twelve mAbs specific for rat MPR-300 (both cloned and uncloned) were then assessed using the Clone 9 hepatocyte cell line. All appeared to cause loss of MPR-300 from a subpopulation of the cells (data not shown), however this ability was shared by an isotype control antibody. Since the purified mAb 1G7/9H4 had no such effect, this was evidently due to some other component



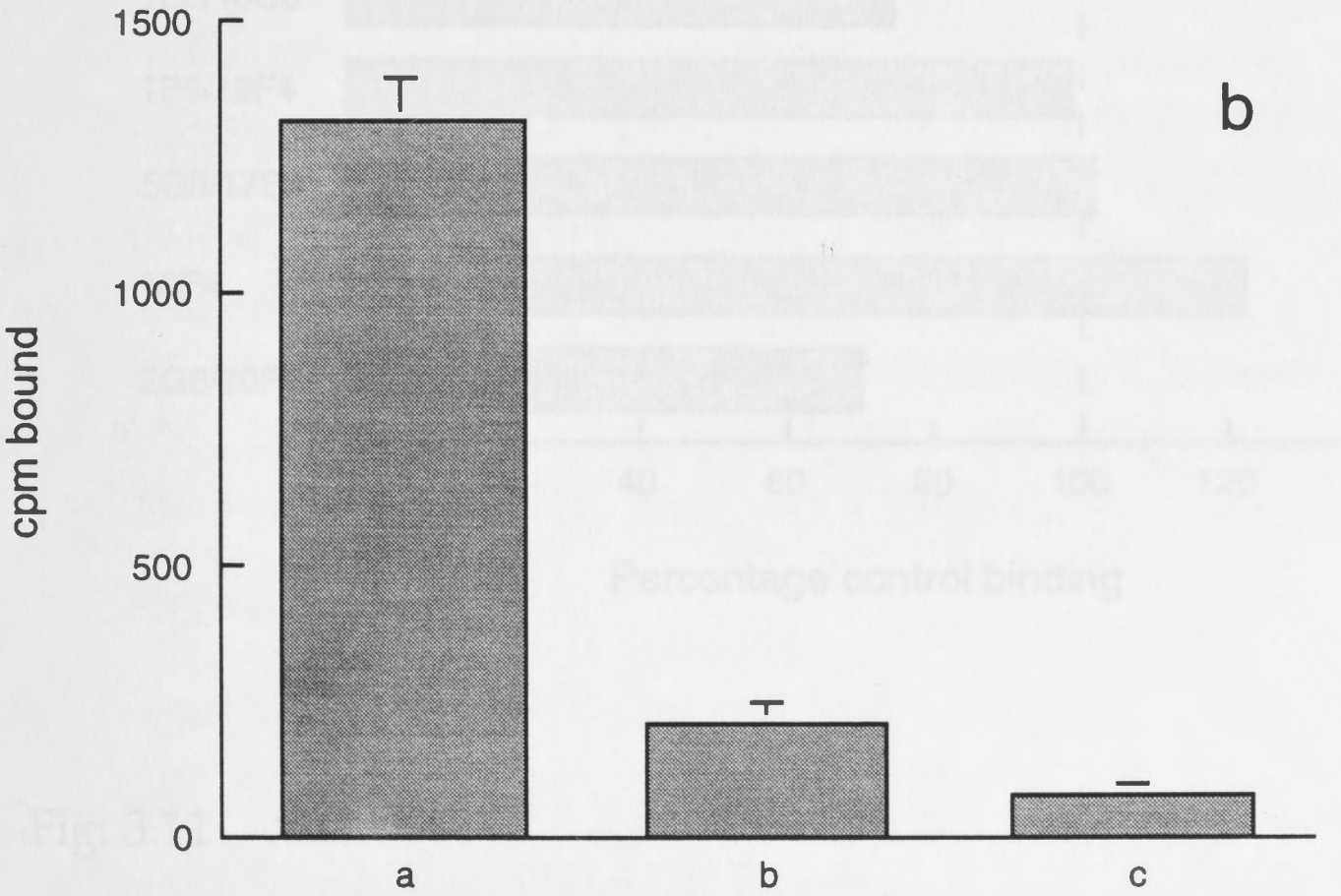


Fig. 3.10

(a) Concentration dependence of ^{125}I - IGF-II binding to purified MPR immobilized on a PVC plate. Values represent single data points.

(b) Binding of 1 nM ^{125}I - IGF-II to immobilized MPR (a), in the presence of 100 nM unlabelled IGF-II (b), and in the absence of MPR (c). Values are mean ± SEM (n=3).

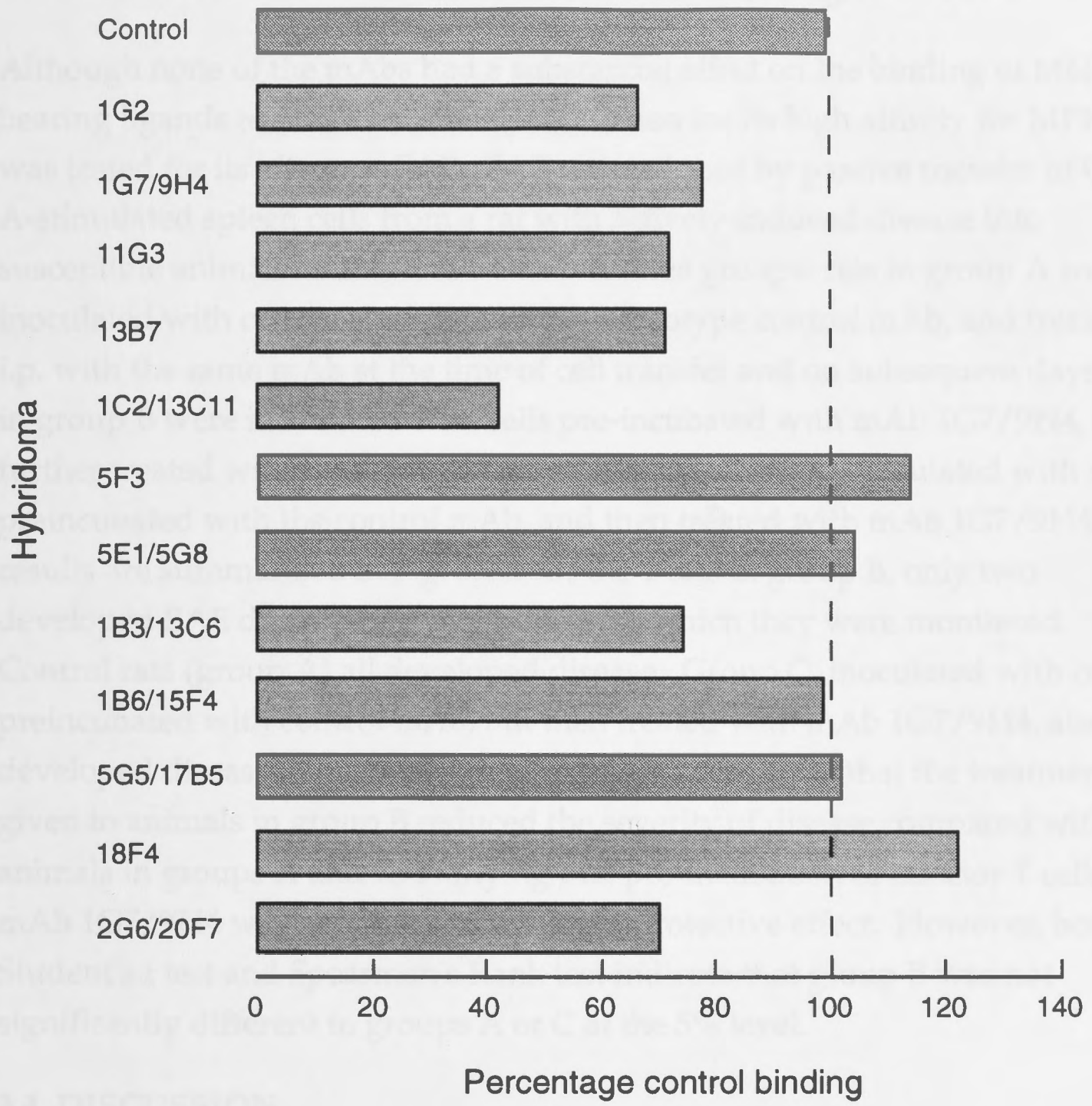


Fig. 3.11

Ability of different anti-MPR mAbs to inhibit binding of 1 nM ^{125}I-IGF-II to purified MPR immobilized on PVC. The dotted line represents ^{125}I -IGF-II binding to MPR in the absence of antibody. Results are expressed as a percentage of this, and are the means of duplicate samples.

of spent hybridoma medium. Testing combinations of mAbs would thus have required purification of each one. This was not done.

3.3.5 The effect of mAb 1G7/9H4 on clinical signs of EAE

Although none of the mAbs had a substantial effect on the binding of M6P-bearing ligands to MPR-300, one mAb, chosen for its high affinity for MPR-300, was tested for its effect on EAE. EAE was induced by passive transfer of Con A-stimulated spleen cells from a rat with actively-induced disease into susceptible animals. Rats were treated in three groups: rats in group A were inoculated with cells preincubated with an isotype control mAb, and treated i.p. with the same mAb at the time of cell transfer and on subsequent days; rats in group B were inoculated with cells pre-incubated with mAb 1G7/9H4, and further treated with the same mAb; rats in group C were inoculated with cells preincubated with the control mAb, and then treated with mAb 1G7/9H4. The results are summarized in Fig. 3.12. Of the 4 rats in group B, only two developed EAE during the 8 day period for which they were monitered. Control rats (group A) all developed disease. Group C, inoculated with cells preincubated with control mAb, but then treated with mAb 1G7/9H4, also all developed disease. The data shown in Fig. 3.12 suggests that the treatment given to animals in group B reduced the severity of disease compared with animals in groups A and C, implying that pre-incubation of effector T cells with mAb 1G7/9H4 was necessary to achieve a protective effect. However, both Student's t test and Spearman's Rank test indicate that group B was not significantly different to groups A or C at the 5% level.

3.4 DISCUSSION

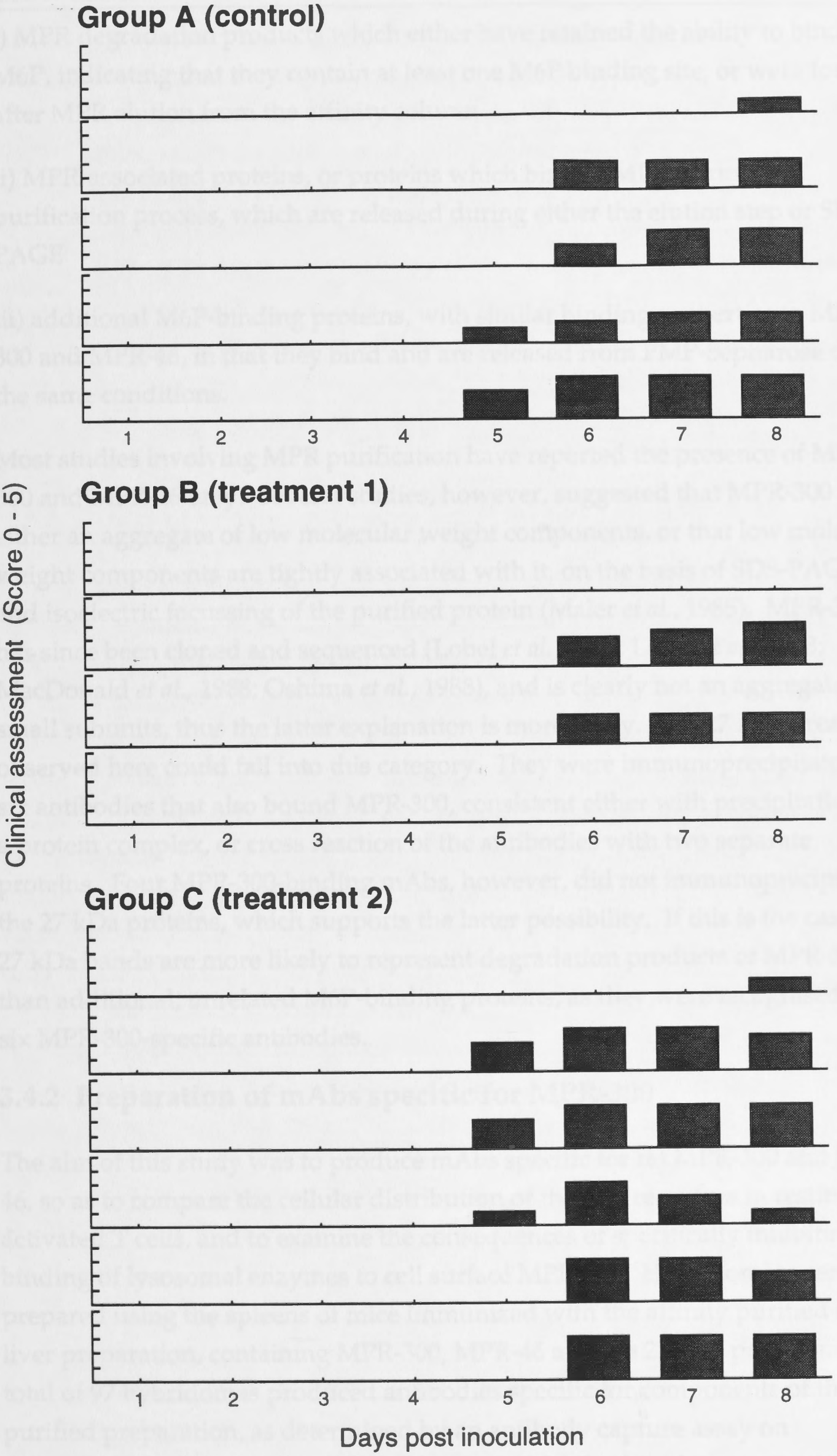
3.4.1 Purification of MPR-300 and MPR-46

MPR-300 and MPR-46 were purified by affinity chromatography on the basis of their ability to bind M6P at pH 6.5, and release it at an acidic pH. Three protein bands were apparent by SDS-PAGE. The two bands of high and intermediate M_r were assumed to be MPR-300 and MPR-46, respectively. MPR-46 appeared as a diffuse, broad band, reflecting the high oligosaccharide content of this protein. Although it's apparent M_r was below 46, 000, the glycosylated form of MPR-46 has been observed to vary in size depending on the tissue source, with 43, 000 previously reported for the rat liver receptor (Section 1.11.3.2).

The affinity purified preparation contained two additional proteins, both with M_r of approximately 27, 000 by SDS-PAGE. These bands may represent:

Fig. 3.12

Effect of mAb 1G7/9H4 on passively-transferred EAE. Female Lewis rats were injected i.v. with Con A stimulated cells prepared from the spleen of a rat immunized ten days previously with 25 μg MBP in FCA. Prior to inoculation, cells were incubated with mAb 1G7/9H4 (group B) or an isotype control mAb (groups A and C). Rats were then injected i.p. with 6 mg/kg of mAb 1G7/9H4 (groups B and C) or isotype control mAb (group A) at the time of cell transfer and on days 1, 2, 3, 4 and 6 post inoculation. Clinical profiles of individual rats are shown, where severity of EAE was scored on an arbitary scale from 0 to 5.


Group A (control)
1
2
3
4
5
6
7
8
Group B (treatment 1)
1
2
3
4
5
6
7
8
Clinical assessment (Score 0 - 5)
Group C (treatment 2)
1
2
3
4
5
6
7
8
Days post inoculation

i) MPR degradation products which either have retained the ability to bind M6P, indicating that they contain at least one M6P binding site, or were formed after MPR elution from the affinity column

ii) MPR-associated proteins, or proteins which bind to MPR during the purification process, which are released during either the elution step or SDS-PAGE

iii) additional M6P-binding proteins, with similar binding properties to MPR-300 and MPR-46, in that they bind and are released from PMP-Sepharose under the same conditions.

Most studies involving MPR purification have reported the presence of MPR-300 and MPR-46 only. Several studies, however, suggested that MPR-300 is either an aggregate of low molecular weight components, or that low molecular weight components are tightly associated with it, on the basis of SDS-PAGE and isoelectric focussing of the purified protein (Maler *et al.*, 1985). MPR-300 has since been cloned and sequenced (Lobel *et al.*, 1987; Lobel *et al.*, 1988; MacDonald *et al.*, 1988; Oshima *et al.*, 1988), and is clearly not an aggregate of small subunits, thus the latter explanation is more likely. The 27 kDa proteins observed here could fall into this category. They were immunoprecipitated by six antibodies that also bound MPR-300, consistent either with precipitation of a protein complex, or cross reaction of the antibodies with two separate proteins. Four MPR-300-binding mAbs, however, did not immunoprecipitate the 27 kDa proteins, which supports the latter possibility. If this is the case, the 27 kDa bands are more likely to represent degradation products of MPR-300 than additional, unrelated M6P-binding proteins, as they were recognised by six MPR-300-specific antibodies.

3.4.2 Preparation of mAbs specific for MPR-300

The aim of this study was to produce mAbs specific for rat MPR-300 and MPR-46, so as to compare the cellular distribution of the two receptors in resting and activated T cells, and to examine the consequences of specifically inhibiting the binding of lysosomal enzymes to cell surface MPR-300. Hybridomas were prepared using the spleens of mice immunized with the affinity purified rat liver preparation, containing MPR-300, MPR-46 and the 27 kDa proteins. A total of 97 hybridomas produced antibodies specific for components of the purified preparation, as determined by an antibody capture assay on immobilized MPR. Of 16 hybridomas selected for further characterization, 12

bound to the cell surface of Clone 9 hepatocytes, which express both MPR-300 and MPR-46, indicating a specificity for the extracytoplasmic domain of either receptor. None of these antibodies, however, bound to the cell surface of McA RH7777 cells, which do not express MPR-300, suggesting that none were specific for MPR-46. This was confirmed by immunoprecipitation of the affinity purified preparation, as the antibodies precipitated MPR-300, but none bound MPR-46. Evidently, MPR-300 was the most immunogenic component of the receptor preparation. In order to generate antibodies to MPR-46, it may be necessary to separate the two receptors, and immunize with MPR-46 alone. Lack of an antibody to MPR-46 precludes a comparison of the expression of this receptor in resting and activated lymphocytes; subsequent chapters will consider activation-related changes in expression of MPR-300 only.

3.4.3 Effect of mAbs on ligand binding to MPR-300

The second reason for producing mAbs to MPR-300 was to provide a means of specifically inhibiting the interaction between cell surface MPR-300 and extracellular lysosomal enzymes, so as to assess the contribution made by cell surface-expressed lysosomal enzymes to basement membrane degradation. The 12 hybridomas with specificity for MPR-300 were examined for their ability to interfere with binding of M6P-bearing ligands, using the assay described in Chapter 2. Three mAbs showed a slight effect (<10% inhibition) on the binding of fluorescein-labelled PPME, a large, M6P-rich polysaccharide, to the cell surface of Clone 9 hepatocytes, and a further three inhibited it's receptor-mediated endocytosis by approximately 20%. Only one mAb, however, inhibited both binding and uptake (1B6/15E4). The poor reproducibility between the binding and uptake assays, and the low level of inhibition exhibited by mAb 1B6/15E4 suggested that none of these antibodies interfered with ligand binding in a biologically significant manner. Prior ligand binding to the receptor also had little effect on binding of the mAbs. The small, monovalent PMP inhibited the binding of 4 antibodies by 20-40%, while the large, multivalent PPME inhibited binding of 5 antibodies by 30-50%. Only 2 mAbs (1C2/13C11 and 5G8), both in different epitope groupings, were affected by both ligands, and neither of these inhibited ligand binding by more than 10%. Together, the two sets of experiments suggest that the ligand and antibody binding sites do not lie close enough on the receptor surface to seriously interfere with one another. As no mAbs showed a clear ability to inhibit ligand binding to MPR-300, it was not possible to pursue a study of the

consequences of neutralizing this interaction on the ability of T cells to induce EAE or to degrade basement membrane components.

Production of antibodies with specificity for the M6P binding site of rat MPR-300 may be difficult to achieve in such closely related a species as the mouse. The human, bovine, rat and murine receptor sequences are known to share 80-90% sequence homology (Ludwig et al., 1994; Oshima et al., 1988), and while the murine and rat sequences have not been directly compared, they may share greater homology than this. The M6P-binding site itself is also likely to be conserved between species. Thus, similarity in the protein structures of rat and murine MPR-300 in the vicinity of the M6P binding site may be too great to generate strongly binding antibodies to this region. Examination of the remaining hybridoma supernatants, particularly those with lower affinity for MPR-300, may yield some that inhibit ligand binding. Alternatively, generation of polyclonal antibodies in a more distantly related species may be more successful. The possiblity also remains that binding of mAbs to cell surface MPR-300, alone or in combination, may lead to a loss of receptors from the cell surface as was described by Nolan *et al.* (1987).

3.4.4 The effect of a MPR-300-specific mAb on passively-transferred EAE

In a preliminary experiment, the effect of one mAb on passively-transferred EAE was examined, despite its inability to inhibit ligand binding. Inspection of the raw data suggested that treating rats with mAb 1G7/9H4 after transfer of MBP-specific T cells did not reduce disease, but that preincubating the effector T cells with mAb 1G7/9H4 before transfer into recipient rats lessened disease severity. The experiment did not include a group in which T cells were pretreated with mAb 1G7/9H4 and recipient rats received control mAb, which might have indicated whether preincubating the cells with antibody was itself sufficient to inhibit disease. The difference between the group inoculated with mAb-pretreated T cells (group B), and the two control groups (A and C) was not statistically significant, however, so speculation about whether binding of mAbs to MPR-300 in the preincubation step merely targeted them for destruction by the immune system, or whether it actually had an effect on T cell migration into the CNS, is inappropriate at this stage. The experiment is, however, suggestive that mAbs with specificity for MPR-300 may inhibit inflammation in EAE, and as such is worth repeating, possibly with different mAbs. MAb 1G7/9H4 was selected on the basis on its high affinity for MPR-300, however, the two mAbs whose binding was inhibited slightly by prior

binding of PMP and PPME could be tested also. Use of antibody $F(ab)_2$ fragments would also exclude the possibility of antibody-dependent cellular cytotoxicity directed towards the MBP-reactive T cells.

3.5 SUMMARY

Lysosomal enzymes expressed on extravasating leukocytes by means of cell surface MPR-300 have been proposed to participate in degradation of the subendothelial basement membrane. As activated T cells display more invasive behaviour than do resting lymphocytes, cell surface expression of lysosomal enzymes is proposed to be enhanced by cellular activation. This may be effected by an activation-induced redistribution of the existing MPR pool, such that MPR-300 is increased at the cell surface, providing more binding sites for extracellular enzymes, with or without an increase in intracellular MPR-46, which is involved in the secretion of newly synthesized lysosomal enzymes, and so could increase their concentration in the extracellular space. In order to compare expression of MPR-300 in resting and activated T cells, and investigate the contribution of MPR-associated lysosomal enzymes to the degradative phenotype, monoclonal antibodies specific for rat MPR-300 were prepared, and assessed for their ability to neutralize binding of extracellular lysosomal enzymes to cell surface MPR-300.

MPR-300 and MPR-46 were purified from rat liver by affinity chromatography on PMP-Sepharose 4B, and a mixture of the two receptors used to immunize BALB/c mice. A total of 97 hybridomas produced antibodies specific for components of the purified MPR preparation, as determined by an antibody capture assay on immobilized MPR, and 16 were selected for further characterization. Twelve of these antibodies bound to MPR in the context of the cell surface of Clone 9 hepatocytes, indicating their specificity for the extracytoplasmic domain of the receptor. None bound to the cell surface of McA RH7777 cells, which lack MPR-300, suggesting that none were specific for MPR-46. This was confirmed by immunoprecipitation of the affinity purified preparation; the antibodies precipitated MPR-300, but none bound MPR-46. Antibodies recognised at least 4 different epitopes on MPR-300, and one or two hybridomas from each group (seven in total) were cloned by limiting dilution. Clones of one hybridoma produced IgM antibodies, while the remainder produced IgG_1.

The 12 hybridomas (seven cloned and five uncloned) were examined for their ability to interfere with binding of M6P-bearing ligands to MPR-300, using the

assay described in Chapter 2. This was based on the binding or endocytosis of fluorescein-labelled PPME, a large, M6P-rich yeast polysaccharide. Three antibodies showed a slight effect on the binding of PPME to the cell surface of Clone 9 hepatocytes (<10% inhibition). Three antibodies also inhibited PPME uptake by approximately 20%, however only one antibody (IgM 1B6/15E4) inhibited both binding and uptake of PPME. The effect of prior ligand binding to the M6P binding site on MPR-300 recognition by the antibodies was also assessed. The small, monovalent ligand pentamannose phosphate (PMP) inhibited the binding of 4 antibodies by 20-40%, while the large, multivalent PPME inhibited binding of 5 antibodies by 30-50%. Binding of only two antibodies (1C2/13C11 and 5G8) was inhibited by both ligands. Neither of these antibodies affected PPME uptake, however, and only 1C2/13C11 influenced PPME binding (10% inhibition). The lack of reproducibility between the various inhibition studies, and the relatively small effects observed, suggest that none of the 12 antibodies examined interfere with the M6P binding site.

Examination of the ability of the antibodies to deplete MPR-300 from the cell surface showed that such an effect was mediated by a component of spent hybridoma medium, indicating that the antibodies would need to be purified in order to assess their effect. It remains possible that a combination of mAbs may be able to crosslink MPR-300 and effect its disappearance from the cell surface.

As none of the 12 mAbs assessed inhibited the binding of M6P-bearing ligands to MPR-300 to any significant degree, it was not possible to investigate the consequences of neutralizing this interaction on the ability of T cells to induce EAE or degrade ECM components. The effect of one mAb, selected on the basis of high affinity for MPR-300, on passively induced EAE was assessed, however results were inconclusive. Although lack of suitable mAbs precluded this aspect of the study, reagents were produced to allow a comparison of MPR distribution in resting and activated cells. No mAbs were specific for MPR-46, however the preparation of mAbs specific for MPR-300 will facilitate the study of activation-related changes in leukocyte expression of this receptor.

CHAPTER 4

A COMPARISON OF MPR-300 EXPRESSION ON THE CELL SURFACE OF RESTING AND ACTIVATED T LYMPHOCYTES

4.1 INTRODUCTION

Activated T lymphocytes appear to be more active than resting cells in their ability to leave the circulation and enter extravascular tissues, as T cell blasts more readily enter the CNS (Hickey *et al.*, 1991; Ludowyk *et al.*, 1992), and are required for the adoptive transfer of EAE (Richert *et al.*, 1979). This can in part be explained by the increased adhesiveness of activated lymphocytes for vascular endothelium, their increased ability to transmigrate through the endothelial cell monolayer (Oppenheimer-Marks *et al.*, 1990; Savion *et al.*, 1984), and their enhanced locomotor capacity (Parrott and Wilkinson, 1981; Ratner *et al.*, 1988; Wilkinson, 1986). However, the ability of T cells to induce EAE has also been correlated with heparanase secretion (Fridman *et al.*, 1987; Naparstek *et al.*, 1984; Savion *et al.*, 1984; Vettel *et al.*, 1991), suggesting that an ability to degrade the subendothelial basement membrane may also contribute to the invasive phenotype. As Parish *et al.* (1990) have proposed that lysosomal enzymes expressed at the cell surface of extravasating leukocytes participate in basement membrane degradation, an increase in the MPR-300-mediated expression of lysosomal enzymes on the surface of activated T cells would be consistent with this model. In Section 1.14, a scenario was presented by which this might occur, thus T cell activation could lead to increased cell surface expression of MPR-300, increased intracellular expression of MPR-46, or both. This study aimed to investigate this question by comparing MPR-300 expression in resting and activated T cells.

MPR-300 and MPR-46 have been detected in almost all cell types studied, the exceptions being several tumour-derived cell lines, such as Morris hepatoma 7777 cells and the macrophage line P388D1, both of which express only MPR-46 (Gabel *et al.*, 1983; Hoflack and Kornfeld, 1985a; Stein *et al.*, 1987d). The bulk of both MPR-300 and MPR-46 is found on intracellular membranes, predominantly in the Golgi cisternae, *trans*-Golgi network and endosomes, with 10% or less on the cell surface (Bleekemolen *et al.*, 1988; Braulke *et al.*, 1987; Brown *et al.*, 1984; Brown and Farquhar, 1984a; Brown and Farquhar, 1984b; Fischer *et al.*, 1980a; Fischer *et al.*, 1980b; Geuze *et al.*, 1984a; Geuze *et al.*, 1985; Geuze *et al.*, 1988; Griffiths *et al.*, 1988; Griffiths *et al.*, 1990; Jin *et al.*, 1989; Kaplan *et al.*, 1977a; Prydz *et al.*, 1990; Stein *et al.*, 1987d; Willingham *et al.*, 1983; Willingham *et al.*, 1981). Most studies involving MPR localization have concentrated on fibroblasts (Braulke *et al.*, 1987; Fischer *et al.*, 1980a; Kaplan *et al.*, 1977a), CHO cells (Willingham *et al.*, 1983; Willingham *et al.*, 1981) and tissues and cell lines of hepatic (Brown *et al.*, 1984; Geuze *et al.*, 1984a; Geuze *et*

al., 1985; Geuze *et al.*, 1988; Stein *et al.*, 1987d) and renal origin (Griffiths *et al.*, 1988; Griffiths *et al.*, 1990; Prydz *et al.*, 1990). Only a few studies have addressed the question of MPR expression by leukocytes. MPR-300 has been demonstrated in rabbit alveolar macrophages, with an estimated 15-20% on the plasma membrane (Shepherd *et al.*, 1984). The U937 human monocyte-like cell line expresses a relatively large amount of both MPR-300 and MPR-46, with approximately 12% of each on the cell surface (Bleekemolen *et al.*, 1988). Similarly, MPR-300 is present on the cell surface of IM-9 cells, a myeloma-derived cell line with characteristics of B lymphoblasts (Brown *et al.*, 1986) and also on the human erythroleukaemic cell line K562 (Jin *et al.*, 1989). Murine splenic T lymphocytes, however, express a low level of MPR-300 intracellularly with none reported on the cell surface (Olsen *et al.*, 1990). With the exception of the U937 cell line, MPR-46 expression by these cells has not been determined.

T cell activation has been shown to induce changes in the intracellular expression and localization of lysosomal enzymes in murine T cells (Olsen *et al.*, 1990). Activation is a complex process during which cells differentiate from quiescent (G_0) to blast cells, involving extensive changes in cellular architecture. Resting lymphocytes are small (average diameter 5 μm), consisting of a large nucleus surrounded by a thin layer of cytoplasm, and containing few discrete or morphologically distinct organelles. They have few lysosome-like structures, with low levels of lysosomal enzyme activity in the cytosolic fraction (Astaldi *et al.*, 1973; Hirschhorn *et al.*, 1968; Olsen *et al.*, 1990). Stimulation with antigen or mitogen results in a rapid increase in biosynthesis, leading to a substantial expansion and organization of the cytoplasm. Following Con A stimulation, T lymphocytes increase in size (average diameter 12 μm) and acquire highly developed cytoplasmic structures, including an extensive RER, Golgi complex, mitochondria and numerous large vesicles. Large lysosomal structures are apparent, and lysosomal enzyme expression increases, becoming associated with vesicles rather than being diffuse in the cytoplasm (Abraham *et al.*, 1986; Olsen, 1990).

Lysosome biogenesis is accompanied by changes in the amount and intracellular distribution of MPR-300. In resting T cells, small quantities of this receptor are found in the cytoplasm as well as in small cytoplasmic vesicles (Olsen *et al.*, 1990). Olsen and coworkers, however, demonstrated a 2-fold increase in total cellular MPR-300 following Con A stimulation, and found it to be located throughout the Golgi complex and in vesicles of the TGN. An implication in this study was that MPR-300 was detected on the plasma

membrane of Con A stimulated, but not resting, T cells . However, no data for cell surface MPR-300 on T cells has been published.

This chapter further examines the expression of MPR-300 on resting and activated T cells, and for comparison, neutrophils and monocytes, using a mAb selected from the panel produced in Chapter 3. The Parish model predicts an increase in cell surface expression of MPR-300 following activation, which could arise either through increased synthesis of the receptor, or by a redistribution of the existing receptor pool (Section 1.14). In the latter case, intracellular expression of MPR-300 would decrease by an amount corresponding to that diverted to the cell surface, thus it could be informative to compare both cell surface and intracellular MPR-300 in resting and activated lymphocytes. However, as the bulk of MPR-300 is expressed on intracellular membranes, and increased expression due to cell growth is to be expected after activation, a small intracellular decrease would probably be undetectable. Hence, this study focussed on expression of MPR-300 on the T cell surface.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Preparation of peripheral blood leukocytes

Fresh whole blood was obtained by venipuncture from healthy human volunteers (up to 10 ml), and by cardiac puncture from anaethatised Fischer rats (up to 4 ml). Heparin (10 U/ml; Commonwealth Serum Laboratories, Melbourne, Australia) was used to prevent coagulation. Blood was centrifuged at 60xg for 15 min at RT and the platelet-rich plasma removed. The cell pellet was resuspended in PBS containing 10% FCS and re-centrifuged at 550xg for 15 min at RT. The supernatant was discarded, and the upper cell layer, consisting mainly of leukocytes, transferred to a 50 ml plastic centrifuge tube. RBCs were lysed with 30 ml of 0.83% ammonium chloride for 5 min at 37°C. Leukocytes were then pelleted by centrifugation at 200xg for 5 min, resuspended in 5 ml cold PBS containing 10% FCS and counted using a haemocytometer.

4.2.2 Isolation of leukocyte subpopulations

To aid in the identification of leukocyte subsets from rat peripheral blood, populations defined on the basis of forward and side scatter were separated using a FACStar Plus (Becton Dickinson, Mountain View, CA). Leukocytes suspended in PBS were filtered through a fine (60 μm) nylon mesh (Ure Pacific, Switzerland) prior to sorting, and each population was collected into a siliconised glass tube containing 0.5 ml FCS. Cells were pelleted by

centrifugation at 200xg for 10 min and resuspended in 100 µl of the supernatant. Cytocentrifuge smears were prepared by centrifuging cells onto glass microscope slides. Smears were air-dried, fixed and stained with Diff-Quick stain (Lab Aids, Narrabeen, Australia). The components of each population were determined by light microscopy.

4.2.3 Preparation of lymphoid cell suspensions

Female Fischer rats (2-3 months old) were killed by CO_2 asphyxiation and the thymus, spleen, Peyer's Patches, and cervical, mesenteric and peripheral lymph nodes removed and placed in ice-cold HBSS or serum-free RPMI 1640. Single cell suspensions were prepared by mincing the tissues finely, then pressing through a wire sieve with the plunger from a 5 ml plastic syringe. Cell clumps were removed by centrifuging for 8 s and discarding the pellet. RBCs and debris were removed by density separation using a mixture of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and metrazoic acid (Sigma, St. Louis, MO) at a ratio of 9:1 (density = 1.0893 g/ml). Cells suspended in 8 ml of 10% FCS/RPMI 1640 medium were underlaid with 2 ml of the Ficoll-Paque mixture in flat-bottomed tubes (Disposable Products, Technology Park, Australia), and centrifuged at 290xg for 20 min at 20°C. The cells collecting at the interface, as well as the Ficoll-Paque medium above the RBC/dead cell pellet, were collected and diluted with 10% FCS/RPMI 1640. Leukocytes were pelleted by centrifugation at 200xg for 5 min at 4°C, washed once with 0% FCS/RPMI 1640 and counted.

4.2.4 Activation of rat spleen cells with Con A

Female Fischer rats (2-3 months old) were killed by CO_2 asphyxiation and spleens removed using sterile technique. Single cell suspensions were prepared, and RBCs and debris removed as described in Section 4.2.3. Cells were resuspended at a density of 1×10^6 cells/ml in Lymphocyte medium (Table 4.1) supplemented with 2 µg/ml Con A. Cells were cultured at 37°C (10% CO_2) for up to 4 days. When cells were to be cultured for 6 days, medium was replaced on day 3 with Lymphocyte medium supplemented with conditioned medium from Con A stimulated rat spleen cells (15%).

Conditioned medium, containing IL-2, was prepared by stimulating rat spleen cells ($1x10^7$) in 10% FCS/RPMI 1640 supplemented with 5 µg/ml Con A for 4-6 h. Cells were washed twice and resuspended at $5x10^6$ cells/ml in serum-free

Table 4.1

Constituents of lymphocyte culture medium

To 1 litre of DMEM (Gibco #430-1600)
(with 1 g/L D-glucose, L-glutamine, 110 mg/l sodium pyruvate)

Add:

Glucose	4 g
Folic acid	6 mg
L-Asparagine	36 mg
L-Arginine.HCl	116 mg
Sodium bicarbonate	2 g
Penicillin	120 mg
Streptomycin sulfate	200 mg
Neomycin sulfate	200 mg

Before use add (per 500 ml):

5 ml x 200 mM L-glutamine (2 mM final concentration)
5 ml x 100 mM sodium pyruvate (1 mM final concentration)
5 ml x 1 M Hepes, pH 7.4 (10 mM final concentration)
0.5 ml 2-mercaptoethanol
50 ml heat-inactivated FCS

RPMI medium supplemented with 100 µg/ml BSA. After 40 h, the supernatant was collected and stored in aliquots at -20°C.

4.2.5 Activation of human peripheral blood mononuclear cells with Con A

Con A-stimulated human T cells, supplied by Dr H. Warren, were prepared as described by Warren and Pembrey (1981). Whole blood was collected from normal healthy donors (ACT Red Cross Blood Bank, Woden Valley Hospital) into sterile tubes containing preservative-free heparin (Commonwealth Serum Laboratories, Melbourne, Australia). It was diluted with an equal volume of PBS, and centrifuged at 400xg for 10 min at RT. Two thirds of the platelet-rich plasma was removed and an equivalent volume of PBS added. Aliquots (8 ml) of diluted blood were underlaid with 2 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) in flat-bottomed plastic tubes (Disposable Products, Technology Park, SA, Australia), and centrifuged at 400xg for 10 min. The lymphocyte interface was collected, diluted with PBS and centrifuged at 250xg for 7 min. The cells were washed twice more in PBS and resuspended in Eagle's medium (Grand Island Biological Co., NY #F-15) supplemented with 10% FCS, 100 µg/ml penicillin, 100 µg/ml streptomycin, 80 µg/ml neomycin, 24 mM sodium bicarbonate and 0.1 mM 2-mercaptoethanol.

Cells were stimulated by culturing lymphocytes at a density of $1x10^6$ cells/ml in the above medium supplemented with 20 µg/ml Con A (Sigma, St. Louis, MO) at 37°C (10% CO_2). After 4 days, cells were washed three times with medium, and resuspended at a density of $2x10^5$ cells/ml in culture medium supplemented with a concentrate of conditioned medium from PHA-stimulated human tonsil lymphocytes (Warren and Pembrey, 1981). After a further two days in culture, cells were either used, or frozen and stored in liquid nitrogen. Frozen cells were thawed and grown in culture medium, supplemented with tonsil lymphocyte-conditioned medium, for one to two days.

4.2.6 Elicitation of peritoneal exudates

Dehydrated fluid thioglycollate medium (Difco Laboratories, Detroit, MI) was reconstituted at 59.6 g/l in boiling water, sterilised and left in the dark to mature for at least six weeks. Peritoneal exudates were elicited by injecting female Fischer rats (2 months old) i.p. with 7 ml of sterile thioglycollate or mineral oil. After one to six days, the exudate was extracted by peritoneal

lavage using 25 ml of ice-cold HBSS. Cells were harvested by centrifugation at 200xg for 5 min at 4°C and resuspended in 10% FCS/RPMI 1640 medium.

4.3.7 Flow cytometry

Leukocyte subsets in peripheral blood, lymphoid tissues and peritoneal exudates were distinguished by flow cytometry, on the basis of size (forward scatter) and internal granularity (side scatter), as described in Section 4.3.1. Ten thousand cells from each sample were collected, and cells from appropriate subsets analysed by setting polygonal regions around the populations of interest.

Cell surface MPR-300, CD4 and CD8 were detected by immunofluorescent flow cytometry. Cells were stained with a primary antibody: mAb 1G7/9H4 (rat MPR-300), rabbit antiserum cross reactive with human MPR-300 (a gift from Dr G.G. Sahagian), mAb W3/25 (rat CD4), mAb OX8 (rat CD8), mAb MT321 (human CD4) or mAb OKT8 (human CD8). CD45 (OX22), LFA-1 and the IL-2 receptor were also detected, using mAbs supplied by Dr D. Willenborg. Cells were washed three times with 10% FCS/RPMI 1640,then incubated with a saturating concentration of fluorescein-conjugated secondary antibody: sheep anti-mouse Ig $F(ab')_2$ fragment (Silenus, Hawthorn, Australia) or swine anti-rabbit Ig antibody (Dakopatts, Denmark). Incubations were in V-bottomed plastic microtitre plates using $2x10^5$ cells in 40 µl volumes. Both incubations were for 30 min on ice. After the final incubation, cells were washed twice with 10% FCS/RPMI 1640 and once with PBS. Cells were fixed with 1% paraformaldehyde and analysed for fluorescence on a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA) using Lysys II software.

B cells in lymphocyte populations also stained due to cross reaction of fluoresceinated secondary antibodies with Ig present on the B cell surface. Several methods were used to prevent this in tissue preparations containing a large proportion of B cells. During analysis of cells from rat lymphoid tissues, undiluted secondary antibodies were preadsorbed with an equal volume of rat serum overnight at 4°C, removing any specificity for rat Ig. Fresh spleen cells in initial experiments involving Con A activation were double-stained with biotinylated sheep anti-rat Ig antibody (Pierce, Rockford, IL) followed by a streptavidin-phycoerythrin conjugate (PE) (Serotec, Star 4B, Oxford, United Kingdom), in addition to staining with mAb 1G7/9H4 and fluorescein-conjugated secondary antibody. A gate was set on PE-negative cells during analysis, and only these cells were analysed for expression of MPR-300. Fresh

spleen cells in later Con A experiments were depleted of B cells by panning (Section 5.2.1.2).

Intracellular expression of MPR-300 was determined by immunofluorescent flow cytometry of permeabilised cells. Cells were permeabilized with saponin using a modification of the method of Goldenthal *et al.* (1985). Cells were washed once with serum-free RPMI 1640, fixed with 4% paraformaldehyde for 5 min at 4°C, and washed twice with RPMI 1640. Cells were incubated with HBSS containing 0.5% BSA and 0.1% saponin for 30 min at RT. The blocking solution was removed, the cells washed once with 10% FCS/RPMI 1640 containing 0.2% saponin, and resuspended in the same medium at 10^7 cells/ml. Aliquots (20 µl) were placed in the wells of V-bottomed microtitre plates and stained as for cell surface staining, except that all solutions aside from the primary antibody contained 0.1% saponin and all steps were performed at RT. After the final incubation, cells were washed three times with 10% FCS/RPMI 1640 containing 0.1%/saponin, then once with PBS, and fixed with 1% paraformaldehyde.

In order to compare the results of separate experiments, expression of MPR-300 was quantified as an "MPR-300 expression index" (MEI), this being the ratio between the median fluorescence intensity due to MPR-300 staining and that due to an appropriate isotype control antibody. Mean MEI values from replicate experiments involving unstimulated cells were compared with a theoretical MEI of 1.0 (indicating no MPR-300 expression), using Student's t test (Swinscow, 1978). Mean MEI values from stimulated cells were compared with unstimulated control populations. p values below 0.05 were considered statistically significant.

4.3 RESULTS

4.3.1 Identification of peripheral blood leukocyte subsets

In order to analyze MPR-300 expression on different peripheral blood leukocyte populations, leukocytes prepared from human and rat blood were resolved into component populations by flow cytometry on the basis of forward and side light scatter (Givan, 1992; Salzman *et al.*, 1975; Thompson *et al.*, 1986). Fig. 4.1(a) illustrates the characteristic light scatter by lymphocytes, monocytes and neutrophils from human peripheral blood. The three major leukocyte populations in rat peripheral blood are shown in Fig. 4.1(b). While neutrophils were clearly distinguishable in the latter, the monocyte and lymphocyte populations were less well separated than in the human scatter

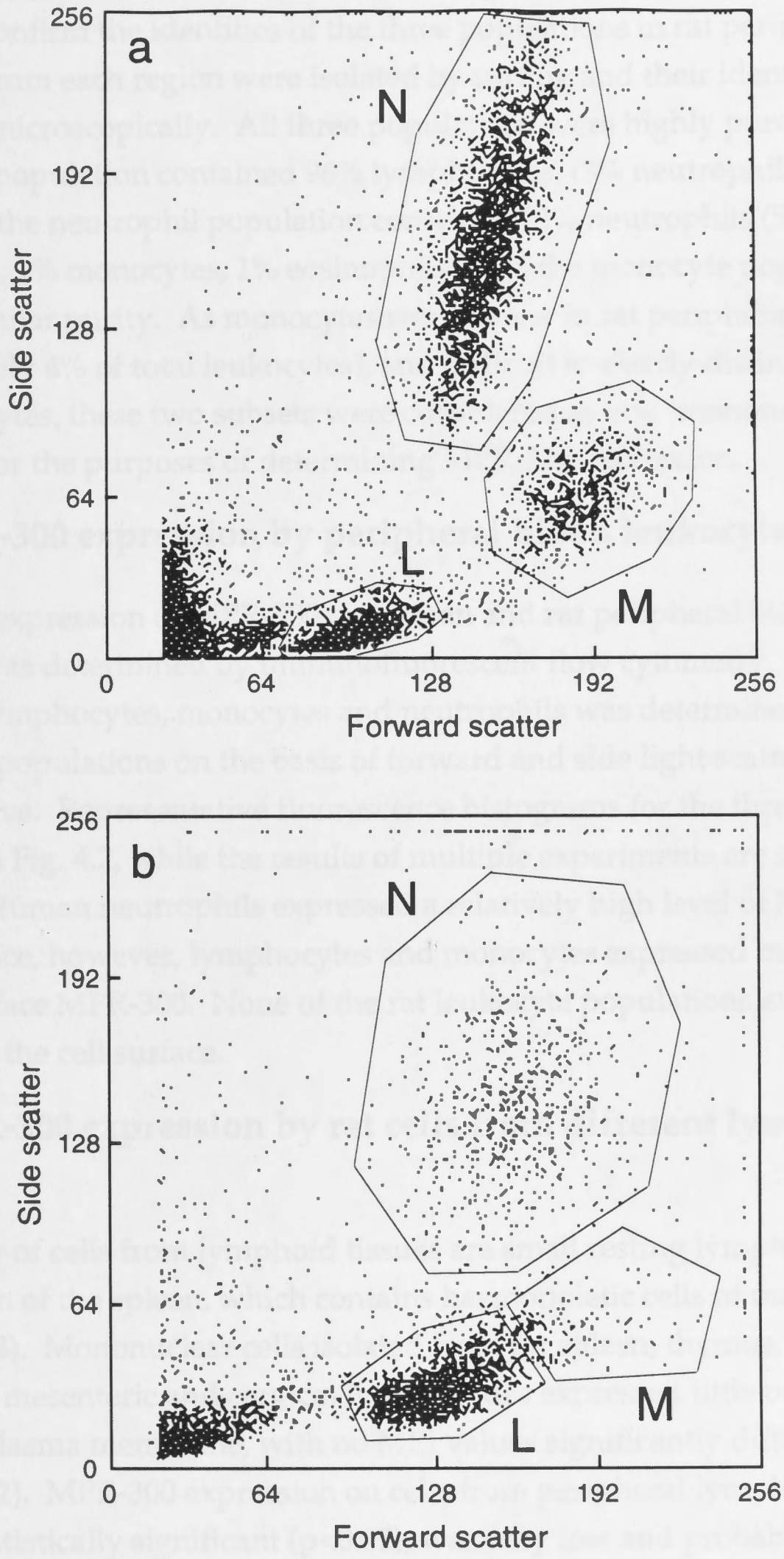


Fig. 4.1

Peripheral blood leukocytes from human (a) and rat (b). Leukocytes were isolated from lysed whole blood and analyzed for forward and side scatter by flow cytometry. Populations identified as lymphocytes, monocytes and neutrophils are designated L, M and N respectively.

profile. To confirm the identities of the three populations in rat peripheral blood, cells from each region were isolated by sorting and their identities determined microscopically. All three populations were highly pure: the lymphocyte population contained 96% lymphocytes, (3% neutrophils and 1% monocytes), the neutrophil population contained 93% neutrophils (5% lymphocytes, 1% monocytes, 1% eosinophils), and the monocyte population exhibited similar purity. As monocytes were so few in rat peripheral blood (approximately 4% of total leukocytes), and difficult to clearly distinguish from the lymphocytes, these two subsets were considered as one "mononuclear cell" population for the purposes of determining MPR-300 expression.

4.3.2 MPR-300 expression by peripheral blood leukocytes

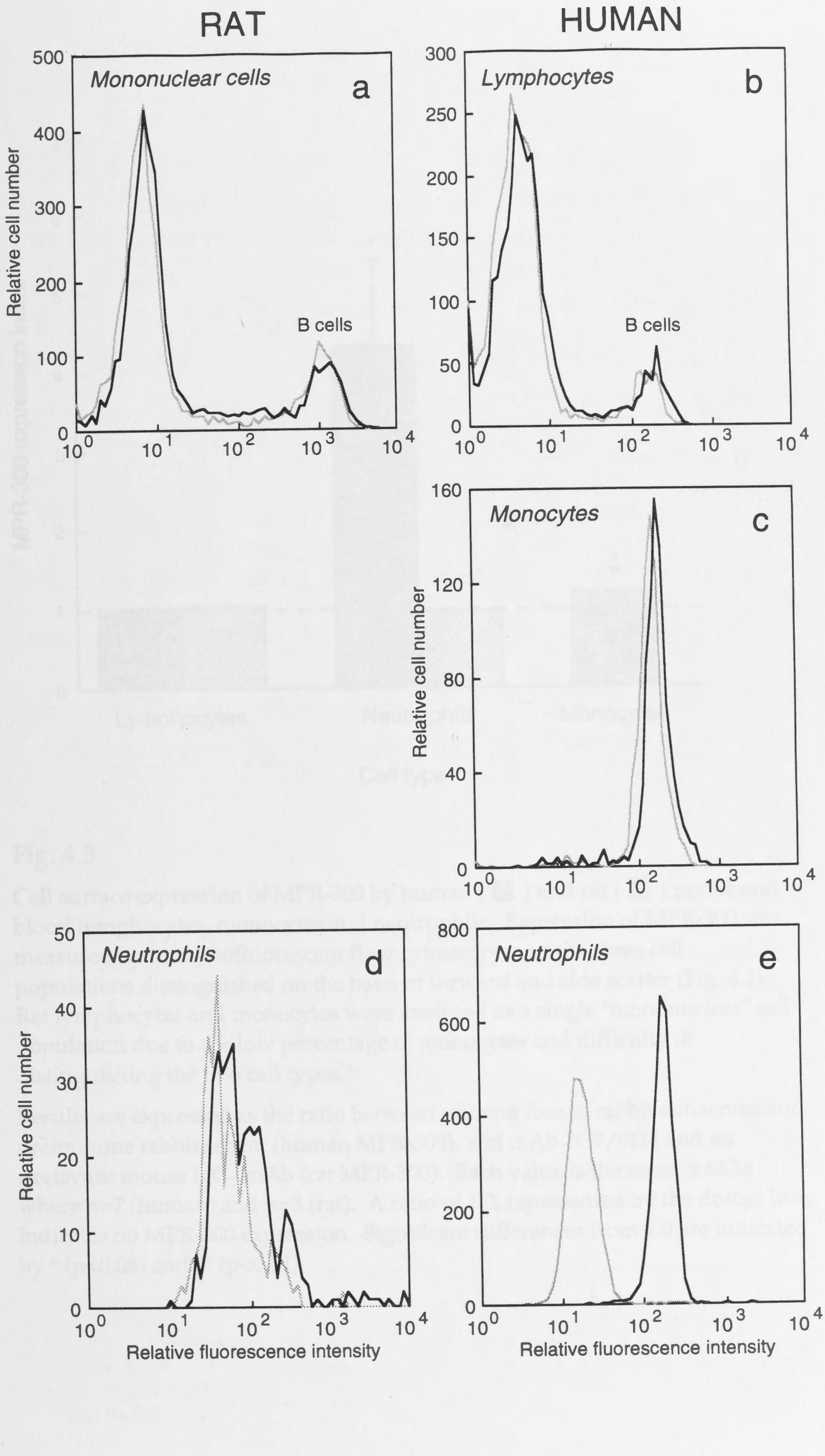
Cell surface expression of MPR-300 on human and rat peripheral blood leukocytes was determined by immunofluorescent flow cytometry. Fluorescent staining of lymphocytes, monocytes and neutrophils was determined by gating appropriate populations on the basis of forward and side light scatter, as outlined above. Representative fluorescence histograms for the three cell types are shown in Fig. 4.2, while the results of multiple experiments are summarised in Fig. 4.3. Human neutrophils expressed a relatively high level of MPR-300 on the cell surface, however, lymphocytes and monocytes expressed minimal, if any, cell surface MPR-300. None of the rat leukocyte populations expressed MPR-300 on the cell surface.

4.3.3 MPR-300 expression by rat cells from different lymphoid organs

The majority of cells from lymphoid tissues are small resting lymphocytes, with the exception of the spleen, which contains haemopoietic cells of many lineages (Sprent, 1993). Mononuclear cells isolated from rat spleen, thymus, Peyer's patches and mesenteric and cervical lymph nodes expressed little or no MPR-300 on the plasma membrane, with no MEI values significantly different from 1.0 (Table 4.2). MPR-300 expression on cells from peripheral lymph nodes, although statistically significant ($p<0.05$), was very low and probably unimportant. Immunofluorescent staining of permeabilized cells showed that all expressed MPR-300 on their intracellular membranes (Table 4.2). Mean MEI values from spleen and thymus cells were statistically significant ($p<0.01$), but due to large variation between replicates, the mean MEI for mesenteric lymph node cells was not. There were insufficient replicates of cells from the peripheral and cervical lymph nodes and Peyer's Patches for statistical analysis.

Fig. 4.2

Cell surface expression of MPR-300 on rat (a, d) and human (b, c, e) peripheral blood lymphocytes, monocytes and neutrophils. Leukocytes were resolved into subpopulations by flow cytometry on the basis of forward and side light scatter (Fig. 4.1). Expression of MPR-300 was measured by immunofluorescent flow cytometry. Binding of rabbit antiserum (human MPR-300) and mAb 1G7/9H4 (rat MPR-300) (——) were compared to staining due to preimmune rabbit serum and an irrelevant mouse IgG_1 mAb, respectively (——). High background fluorescence of monocytes and neutrophils, compared with lymphocytes, was due to non-specific binding of antibodies as autofluorescence of unstained cells was low.


RAT
HUMAN
Mononuclear cells
a
B cells
Relative cell number
Lymphocytes
b
B cells
Monocytes
c
Neutrophils
d
Neutrophils
e
Relative fluorescence intensity
Relative fluorescence intensity

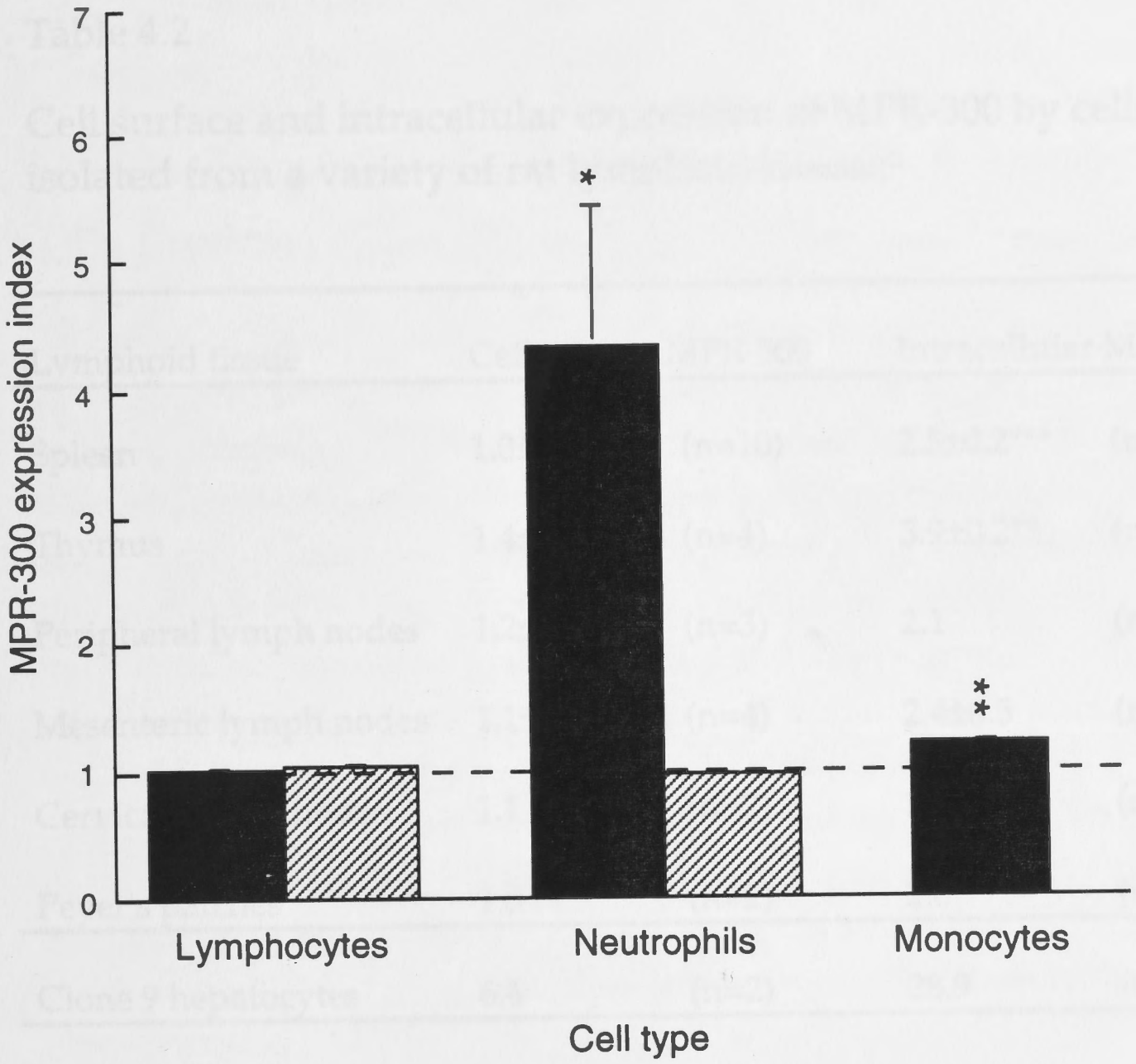


Fig. 4.3

Cell surface expression of MPR-300 by human (■) and rat (▨) peripheral blood lymphocytes, monocytes and neutrophils. Expression of MPR-300 was measured by immunofluorescent flow cytometry, and the three cell populations distinguished on the basis of forward and side scatter (Fig. 4.1). Rat lymphocytes and monocytes were analysed as a single "mononuclear" cell population due to the low percentage of monocytes and difficulty in distinguishing the two cell types.

Results are expressed as the ratio between staining due to rabbit antiserum and preimmune rabbit serum (human MPR-300), and mAb 1G7/9H4 and an irrelevant mouse IgG_1 mAb (rat MPR-300). Each value is the mean ± SEM where n=7 (human) and n=3 (rat). A ratio of 1.0, represented by the dotted line, indicates no MPR-300 expression. Significant differences from 1.0 are indicated by * ($p<0.05$) and ** ($p<0.01$).

Table 4.2

Cell surface and intracellular expression of MPR-300 by cells isolated from a variety of rat lymphoid tissues[a]

Lymphoid tissue	Cell surface MPR-300		Intracellular MPR-300	
Spleen	1.0±0.1	(n=10)	2.5±0.2***	(n=8)
Thymus	1.4±0.2	(n=4)	3.9±0.2**	(n=4)
Peripheral lymph nodes	1.2±0.0*	(n=3)	2.1	(n=2)
Mesenteric lymph nodes	1.1±0.1	(n=4)	2.4±0.5	(n=4)
Cervical lymph nodes	1.1	(n=2)	3.4	(n=2)
Peyer's patches	1.0	(n=2)	2.0	(n=2)
Clone 9 hepatocytes	6.4	(n=2)	28.9	(n=2)

* $p<0.05$

** $p<0.01$

*** $p<0.001$

a Expression of MPR-300 was measured by immunofluorescent flow cytometry, and is expressed as the ratio between staining due to mAb 1G7/9H4 (rat MPR-300) and an irrelevant mouse IgG_1, such that a ratio of 1.0 indicates no MPR-300 expression. Each value is the mean±SEM of the indicated number of samples.

Representative fluorescence histograms (Fig. 4.4) compare MPR-300 expression by spleen and mesenteric lymph node cells with that of the Clone 9 hepatocyte cell line. Cell surface expression of MPR-300 on Clone 9 cells, but not lymphoid cells, is clearly evident. Intracellular MPR-300 expression by all lymphoid cells was detectable but low in comparison with Clone 9 hepatocytes, whose high intracellular expression is due at least in part to a larger cytoplasmic volume.

4.3.4 MPR-300 expression by Con A stimulated T lymphocytes

As described above, freshly isolated lymphocytes from rat spleen expressed no detectable cell surface MPR-300. *In vitro* stimulation with Con A, however, induced expression of MPR-300 on the plasma membrane of T lymphocytes in a time-dependent manner. Representative fluorescence profiles are shown in Fig. 4.5 (a, c, e), and results of multiple experiments are summarised in Fig. 4.6 (a). MPR-300 expression by unstimulated rat splenic T lymphocytes was minimal. Cells were re-examined after 3, 4 and 6 days stimulation with Con A. After 3 days, MPR-300 was present on the cell surface, with a mean MEI value of 2.1±0.4. Its expression was slightly diminished on day 4, and had dropped further by day 6, to a level where it was no longer significantly different from the unstimulated spleen cell population ($p>0.05$). In a single experiment, MPR-300 had not yet appeared after 18 h in culture (data not shown), suggesting that expression of MPR-300 on the cell surface is a relatively late event following exposure to Con A.

T cells from human peripheral blood were stimulated with Con A for 4 days, and then maintained in IL-2 supplemented medium for a further 2 days. At this point they did not express significant amounts of MPR-300 on their plasma membranes (Fig. 4.5 (f), Fig. 4.6 (b)). In a single experiment, a very low level of MPR-300 was detected after 3 days of Con A stimulation (Fig. 4.5 (d)).

4.3.5 Expression of MPR-300 by thioglycollate-elicited rat peritoneal leukocytes

In order to examine expression of MPR-300 on rat leukocytes activated by an *in vivo* stimulus, cells elicited into the peritoneal cavity by thioglycollate broth were compared with resident peritoneal cells. Four cell populations (regions R1 - R4), distinguishable by forward and side scatter, were resident in the peritoneal cavity prior to thioglycollate treatment (Fig. 4.7 (a)). Within 48 h of thioglycollate injection, there was an 8-fold increase in cell number, from an initial level of approximately $14x10^6$ cells. Increases in each of the four resident populations contributed to this, however a fifth population (R5) formed the

Fig. 4.4

Cell surface and intracellular expression of MPR-300 by rat Clone 9 hepatocytes (a, b), and lymphocytes isolated from rat spleen (c, d) and mesenteric lymph nodes (e, f). MPR-300 expression was measured by immunofluorescent flow cytometry: intact and saponin-permeabilized cells were stained with mAb 1G7/9H4 (——) and compared with cells exposed to an irrelevant mouse IgG1 mAb (——).

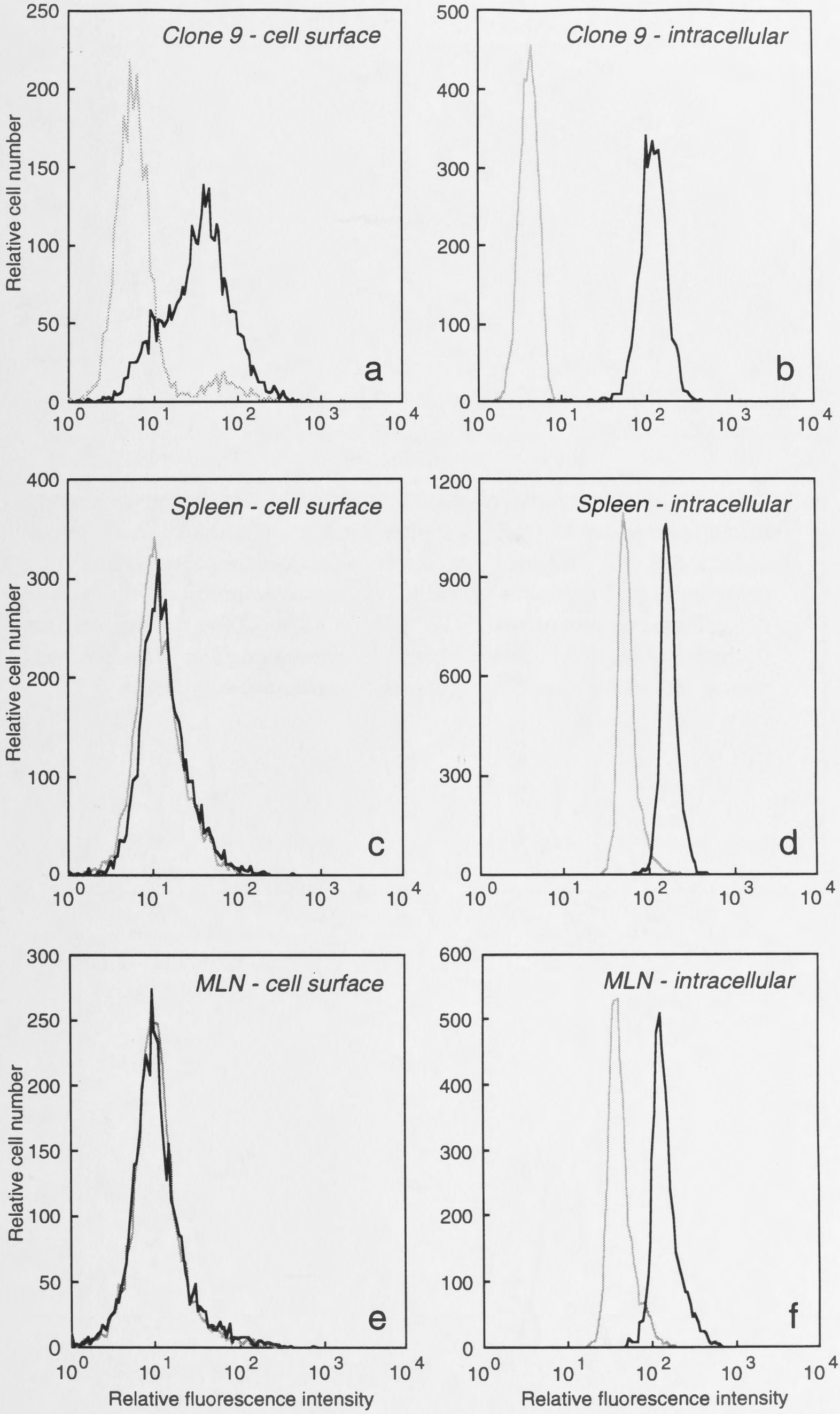
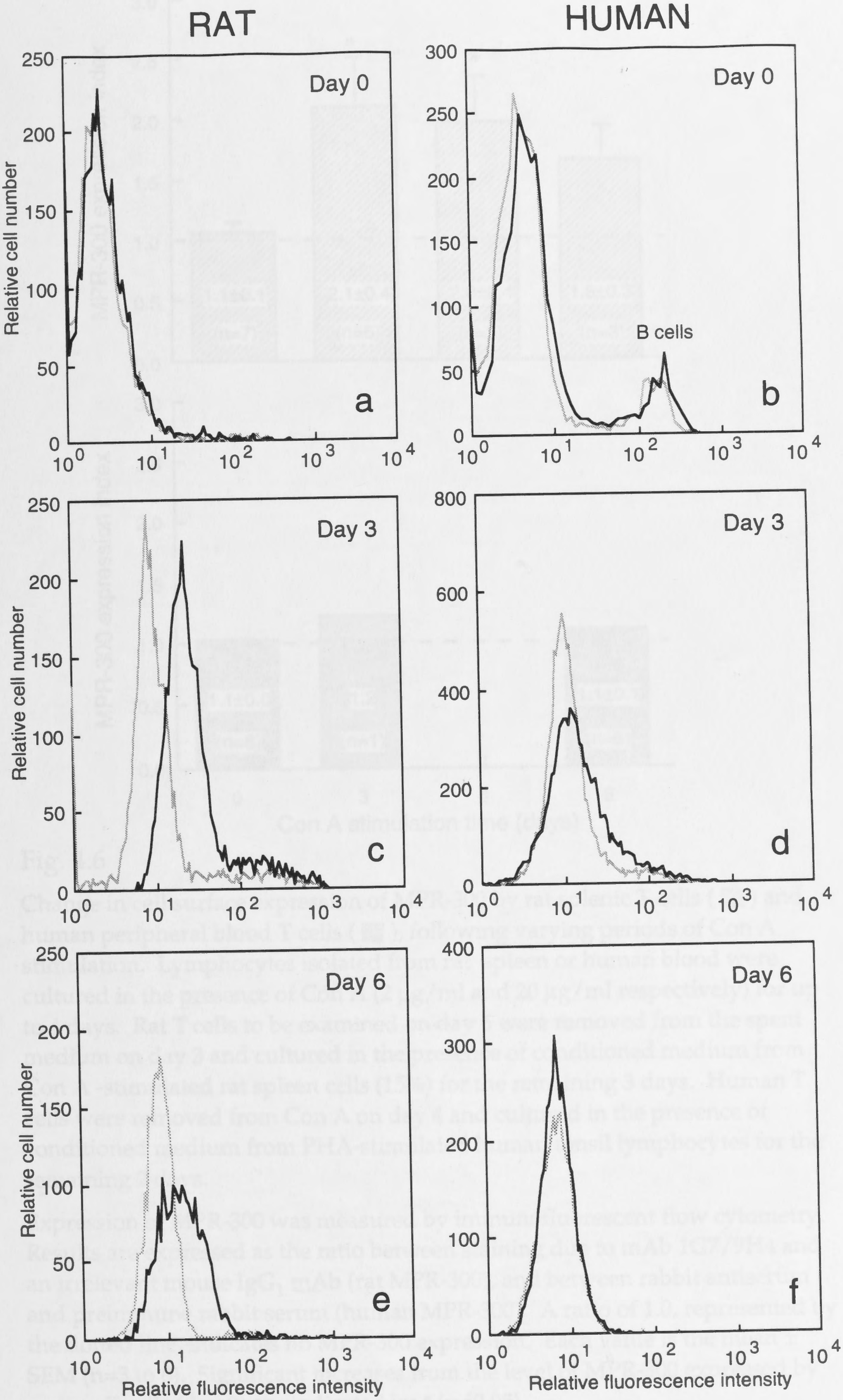

Clone 9 - cell surface
a
Clone 9 - intracellular
b
Spleen - cell surface
c
Spleen - intracellular
d
MLN - cell surface
e
MLN - intracellular
f
Relative cell number
Relative fluorescence intensity
250
200
150
100
50
0
500
400
300
1200
900
600
10^0
10^1
10^2
10^3
10^4

Fig. 4.5

Cell surface expression of MPR-300 by T cells from rat spleen (a, c, e) and human peripheral blood (b, d, f) after stimulation with Con A. Expression of MPR-300 on freshly isolated cells (a, b) and cells exposed to Con A for 3 (c, d) and 6 (e, f) days was measured by immunofluorescent flow cytometry. Binding of mAb 1G7/9H4 (rat MPR-300) or rabbit antiserum (human MPR-300)(——) was compared with staining due to an irrelevant mouse IgG_1 mAb or preimmune rabbit serum, respectively (·····).


RAT
HUMAN
Day 0
Day 0
B cells
a
b
Day 3
Day 3
c
d
Day 6
Day 6
e
f
Relative cell number
Relative fluorescence intensity
Relative fluorescence intensity

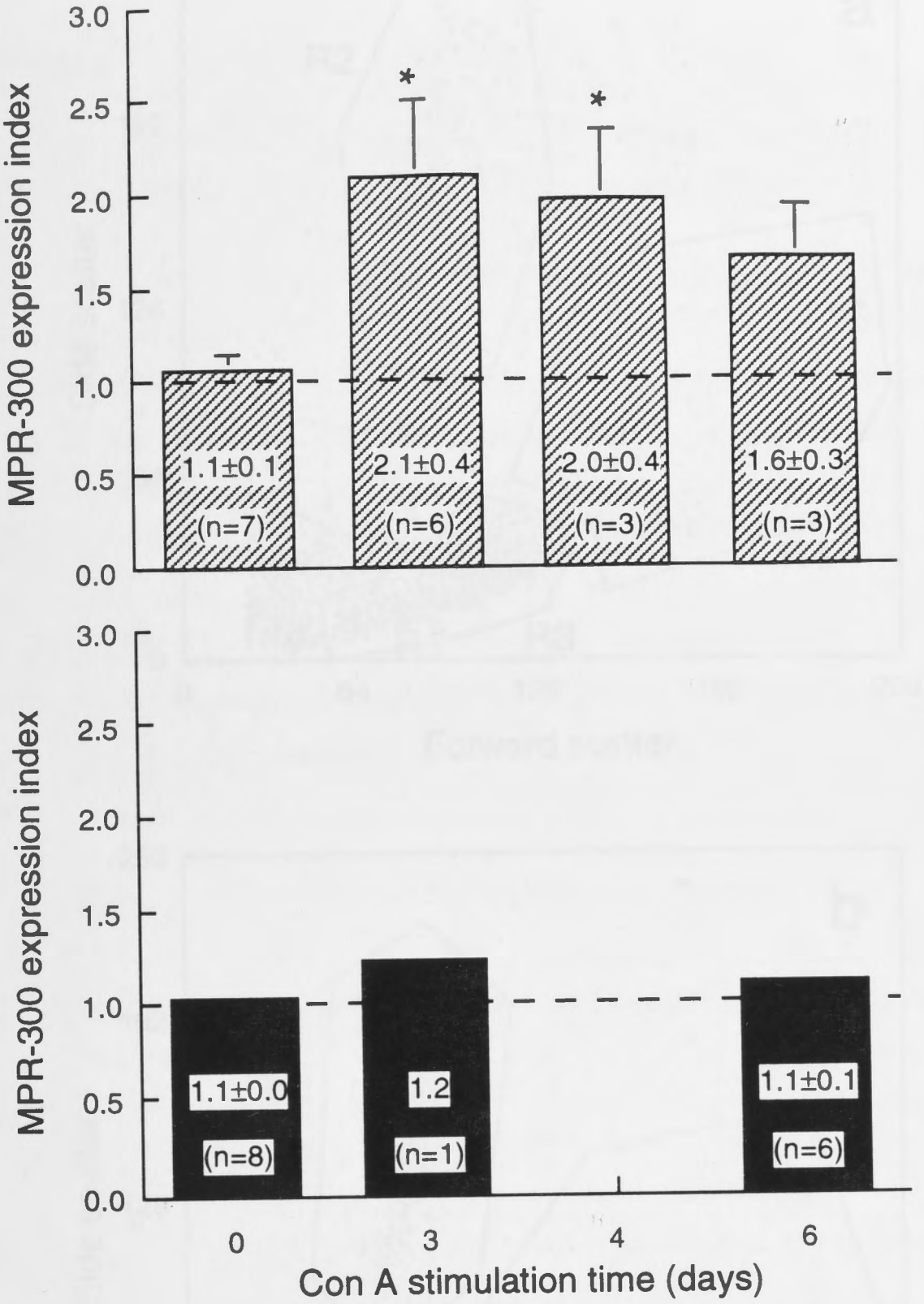


Fig. 4.6

Change in cell surface expression of MPR-300 by rat splenic T cells (▨) and human peripheral blood T cells (■), following varying periods of Con A stimulation. Lymphocytes isolated from rat spleen or human blood were cultured in the presence of Con A (2 μg/ml and 20 μg/ml respectively) for up to 4 days. Rat T cells to be examined on day 6 were removed from the spent medium on day 3 and cultured in the presence of conditioned medium from Con A -stimulated rat spleen cells (15%) for the remaining 3 days. Human T cells were removed from Con A on day 4 and cultured in the presence of conditioned medium from PHA-stimulated human tonsil lymphocytes for the remaining 2 days.

Expression of MPR-300 was measured by immunofluorescent flow cytometry. Results are expressed as the ratio between staining due to mAb 1G7/9H4 and an irrelevant mouse IgG_1 mAb (rat MPR-300), and between rabbit antiserum and preimmune rabbit serum (human MPR-300). A ratio of 1.0, represented by the dotted line, indicates no MPR-300 expression. Each value is the mean ± SEM (n=3 to 8). Significant increases from the level of MPR-300 expressed by resting T cells (day 0) are indicated by * ($p<0.05$).

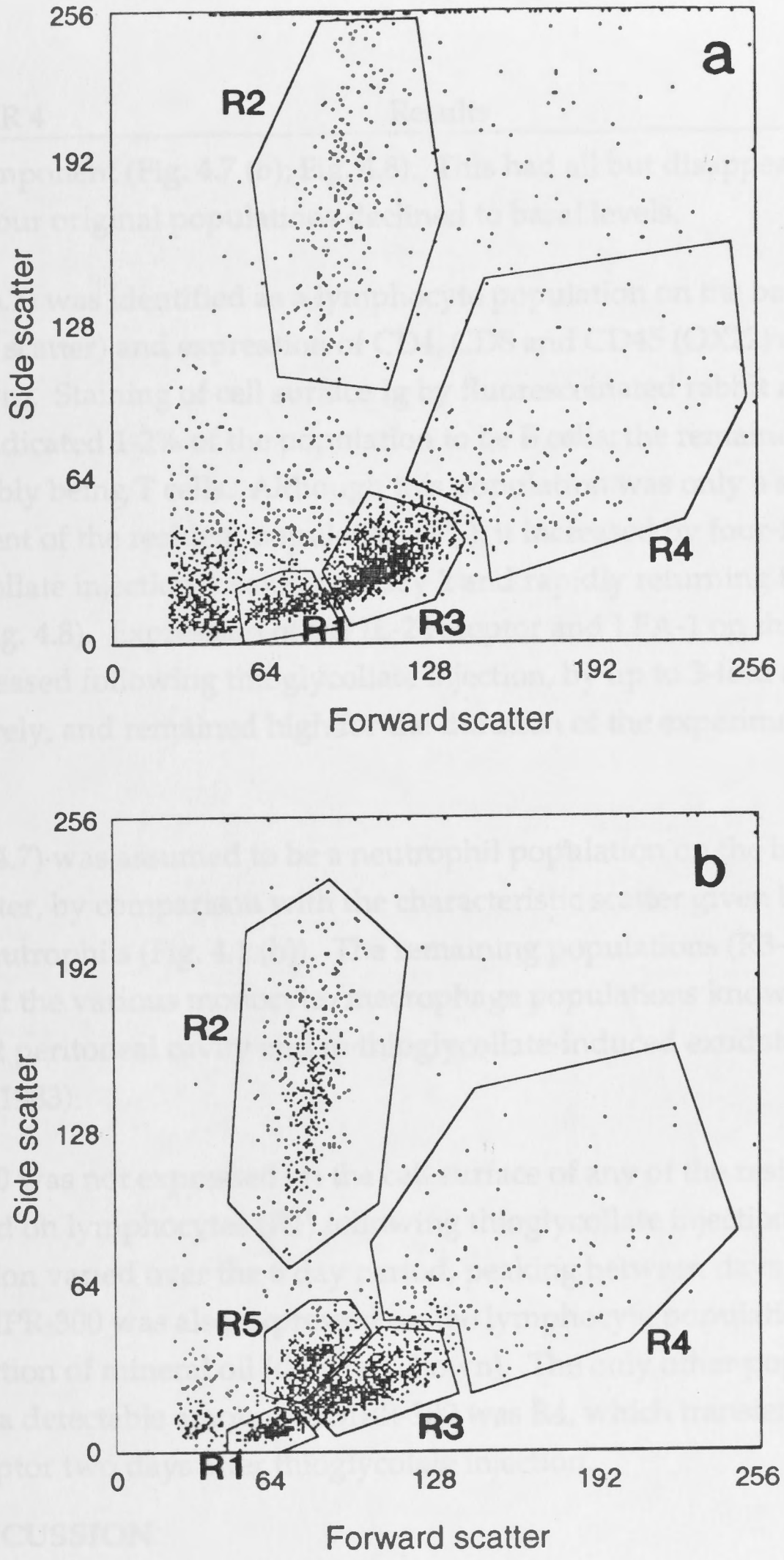


Fig. 4.7

Leukocytes isolated from the peritoneal cavity of an untreated rat (a) and three days after intraperitoneal injection of thioglycollate (b). Leukocytes were harvested by peritoneal lavage and analyzed for forward and side scatter by flow cytometry. Subpopulations are indicated by regions R1 to R5.

major component (Fig. 4.7 (b); Fig. 4.8). This had all but disappeared by day 6, and the four original populations declined to basal levels.

R1 (Fig. 4.7) was identified as a lymphocyte population on the basis of size (forward scatter) and expression of CD4, CD8 and CD45 (OX22) antigens (data not shown). Staining of cell surface Ig by fluoresceinated rabbit anti-mouse Ig $F(ab')_2$ indicated 1-2% of the population to be B cells; the remainder presumably being T cells. Although this population was only a small component of the resident population (3%), it increased by four-fold after thioglycollate injection, peaking on day 2 and rapidly returning to normal levels (Fig. 4.8). Expression of the IL-2 receptor and LFA-1 on this population also increased following thioglycollate injection, by up to 3-fold and 4-fold respectively, and remained high for the duration of the experiment (data not shown).

R2 (Fig. 4.7) was assumed to be a neutrophil population on the basis of its high side scatter, by comparison with the characteristic scatter given by peripheral blood neutrophils (Fig. 4.1 (b)). The remaining populations (R3-R5) probably represent the various monocyte/macrophage populations known to be present in the rat peritoneal cavity and in thioglycollate-induced exudates (Beelen and Walker, 1983).

MPR-300 was not expressed on the cell surface of any of the resident cells, but appeared on lymphocytes (R1) following thioglycollate injection (Fig. 4.9). Expression varied over the 6 day period, peaking between days 3 and 4 (Fig. 4.10). MPR-300 was also expressed on the lymphocyte population 4 days after i.p. injection of mineral oil (data not shown). The only other population to express a detectable amount of MPR-300 was R4, which transiently expressed the receptor two days after thioglycolate injection.

4.4 DISCUSSION

Most cell types examined in earlier studies constitutively express MPR-300 on the plasma membrane (Section 4.1). In contrast, this study showed that MPR-300 is not expressed on the cell surface of unstimulated T lymphocytes, nor on most other leukocytes in the resting state. Rat T cells were found to express MPR-300 on the cell surface in two instances: following *in vitro* stimulation with Con A, and at a site of inflammation.

While most other cell types express up to 10% of total MPR-300 on the plasma membrane (Section 4.1), there was a clear exclusion of this receptor from the

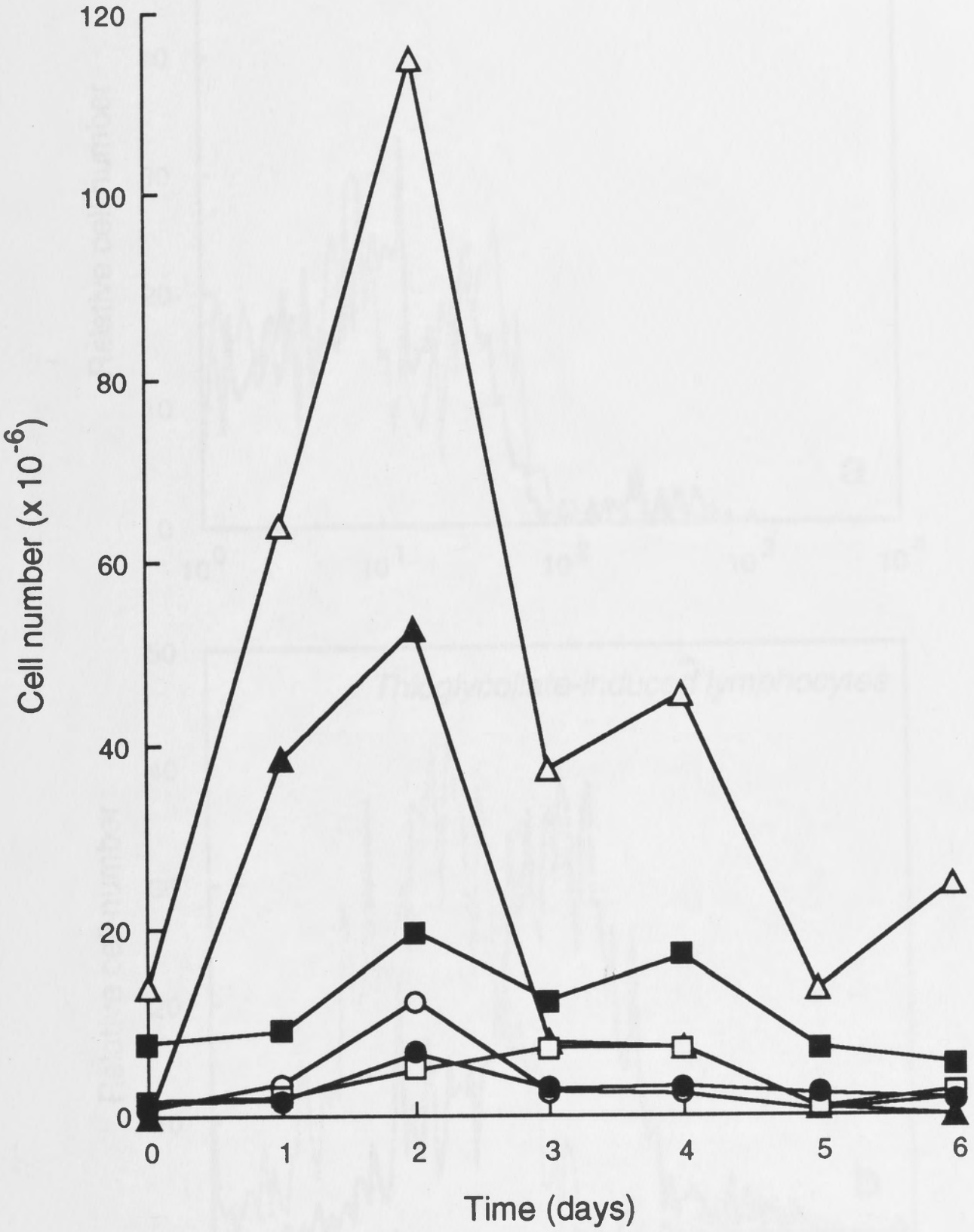


Fig. 4.8

Entry of leukocytes into the rat peritoneal cavity following intraperitoneal injection of thioglycollate. Leukocytes were harvested by peritoneal lavage and analysed by flow cytometry. Subpopulations were distinguished on the basis of forward and side scatter (Fig. 4.7): total cells (△), lymphocytes (●), neutrophils (□), and monocytes/macrophages (R3; ■), (R4; ○) and (R5; ▲). Data points represent single rats.

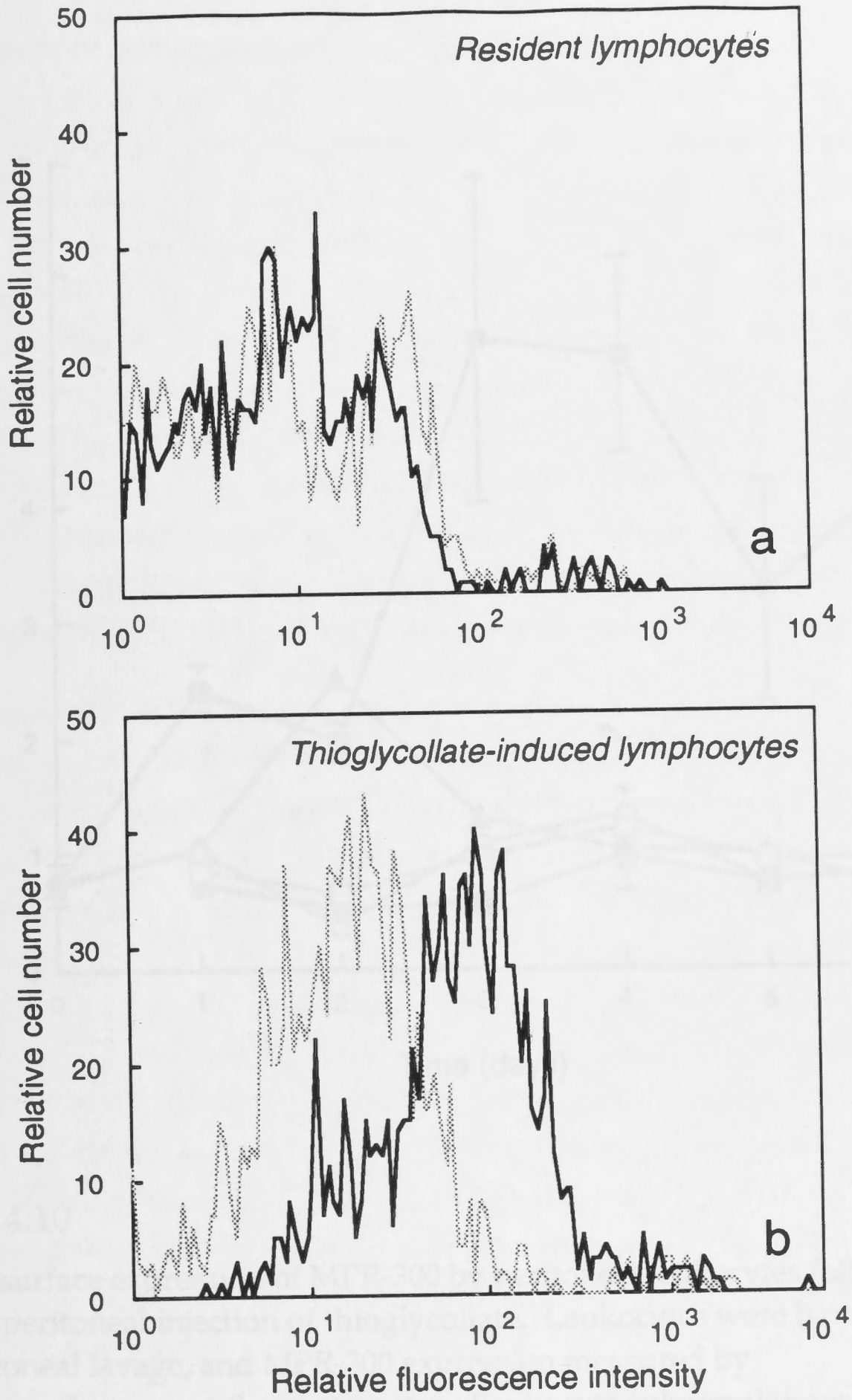


Fig. 4.9

Expression of MPR-300 on peritoneal lymphocytes from an untreated rat (a) and 4 days after intraperitoneal injection of thioglycollate (b). Leukocytes were harvested by peritoneal lavage and expression of MPR-300 measured by immunofluorescent flow cytometry. Binding of mAb 1G7/9H4 (rat MPR) (——) was compared with binding of an irrelevant mouse IgG1 mAb (·····). The lymphocyte population was distinguished on the basis of forward and side scatter (Fig. 4.7).

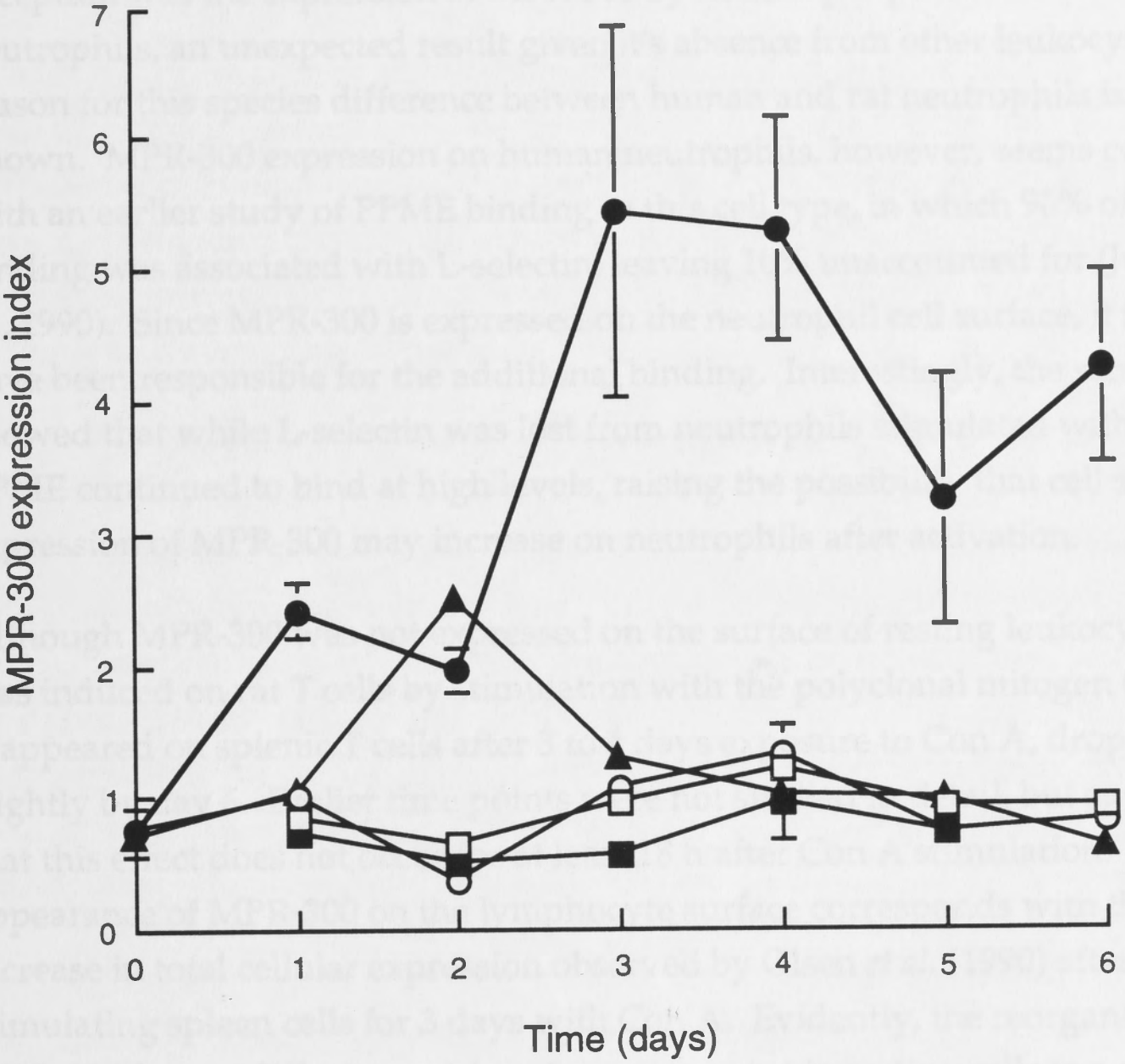


Fig. 4.10

Cell surface expression of MPR-300 by peritoneal leukocytes following intraperitoneal injection of thioglycollate. Leukocytes were harvested by peritoneal lavage, and MPR-300 expression measured by immunofluorescent flow cytometry. Leukocyte subpopulations were distinguished on the basis of forward and side scatter (Fig. 4.7): lymphocytes (●), neutrophils (□), R3 (○), R4 (▲) and R5 (■) are shown. Results are expressed as the ratio between staining due to mAb 1G7/9H4 (rat MPR-300) and an irrelevant mouse IgG_1, such that a ratio of 1.0 indicates no MPR-300 expression. Each value is the mean±SEM of triplicate samples from single rats.

cell surface of leukocytes from peripheral blood and lymphoid tissues, the latter consisting chiefly of small resting lymphocytes (Sprent, 1993). An exception was the expression of MPR-300 by human peripheral blood neutrophils, an unexpected result given it's absence from other leukocytes. The reason for this species difference between human and rat neutrophils is not known. MPR-300 expression on human neutrophils, however, seems consistent with an earlier study of PPME binding to this cell type, in which 90% of binding was associated with L-selectin, leaving 10% unaccounted for (Jutila *et al.*, 1990). Since MPR-300 is expressed on the neutrophil cell surface, it may have been responsible for the additional binding. Interestingly, the same study showed that while L-selectin was lost from neutrophils stimulated with C5a, PPME continued to bind at high levels, raising the possibility that cell surface expression of MPR-300 may increase on neutrophils after activation.

Although MPR-300 was not expressed on the surface of resting leukocytes, it was induced on rat T cells by stimulation with the polyclonal mitogen Con A. It appeared on splenic T cells after 3 to 4 days exposure to Con A, dropping slightly by day 6. Earlier time points were not studied in detail, but suggested that this effect does not occur for at least 18 h after Con A stimulation. The appearance of MPR-300 on the lymphocyte surface corresponds with the increase in total cellular expression observed by Olsen *et al.* (1990) after stimulating spleen cells for 3 days with Con A. Evidently, the reorganization of MPR-300 from a diffuse, cytoplasmic arrangement in resting cells to a more organised distribution through the Golgi and TGN of activated cells (Olsen *et al.*, 1990), includes the cell surface. This change in MPR-300 localization is consistent with its proposed role in the development of EAE. When Con A-stimulated spleen cells from rats immunised with MBP are used to passively transfer EAE, stimulation is usually carried out for 3 days prior to transfer. Con A-activated T cells enter the CNS in less than 24 h (Hickey *et al.*, 1991), indicating that they have acquired a migratory phenotype and are capable of entering the CNS three to four days after initiation of Con A stimulation. This coincides with the time when MPR-300 is present on the plasma membrane, so is compatible with a role for MPR-300 in T cell migration into the CNS.

In contrast to rat splenic T cells, human peripheral blood lymphocytes stimulated with Con A did not express MPR-300. A possible explanation for this will be considered in Chapter 5. Interestingly, a recent study showed that M6P inhibited heparanase-mediated ECM degradation by PMA-stimulated human peripheral blood lymphocytes (Bartlett *et al.*, 1995a), suggesting that

PMA stimulation may induce cell surface expression of MPR-300. If this is the case, MPR-300 must appear very rapidly on human lymphocytes, as this effect was observed over a stimulation period of only 24 h.

While the appearance of MPR-300 on the cell surface of rat T cells stimulated with Con A is consistent with the proposed model of T cell migration, in which activated T cells bind degradative enzymes to their surface via MPR-300, Con A does not provide a physiological activation stimulus. Peritoneal exudate cells were therefore examined to see whether MPR-300 expression could be induced by an *in vivo* activating stimulus.

None of the leukocyte populations present in the peritoneal cavity prior to thioglycollate injection expressed MPR-300 on the cell surface; nor did elicited macrophages and neutrophils. Bartlett *et al.* (1994) recently showed that M6P inhibited thioglycollate-induced entry of leukocytes into the murine peritoneal cavity, exerting its inhibitory effect predominantly on neutrophils, which is intriguing in view of the lack of MPR-300 on inflammatory neutrophils. This may represent yet another species difference, between rat and murine neutrophils. Alternatively, neutrophils may express MPR-300 only transiently during the extravasation process.

Although lymphocytes were only a minor component of the cellular exudate, they alone expressed MPR-300. Increased expression of the IL-2 receptor and LFA-1, both markers of activation (Desroches *et al.*, 1991; Kurzinger *et al.*, 1981; Robb, 1984), indicates that lymphocytes isolated from the inflamed peritoneum were more highly activated than the original population. In addition, other investigators have shown that lymphocytes from peritoneal exudates are capable of migration into inflamed tissues: such cells injected into recipient rats migrate very well into DTH lesions (Issekutz *et al.*, 1986) and arthritic joints (Issekutz and Issekutz, 1991), and can transfer contact sensitivity (Ptak and Asherson, 1969). Expression of MPR-300 by these cells thus appears consistent with our proposed model, as the activated, and potentially migratory, lymphocyte population expressed MPR-300 while the inactive "resident" cells did not.

It is not clear, however, how and where the exudate lymphocytes became activated, at what point MPR-300 appeared on the cell surface, and whether it was involved in their entry into the peritoneal cavity. This leads to the question of whether expression of MPR-300 on the lymphocyte surface is an unrelated consequence of activation, or whether it does in fact contribute to the

capacity of the cell to migrate into extravascular tissues. One possibility is that the activated lymphocyte population in the peritoneal exudate was derived from the "resident" population, rather than from the inflammatory infiltrate. In this case, expression of MPR-300 would reflect activation only, without necessarily having any correlation with the ability of these cells to migrate. Several studies suggest that resident lymphocytes could become activated to some extent as a result of the inflammatory reaction: supernatants from non-immunological inflammatory exudates (both pleural and peritoneal) have been shown to induce DNA synthesis in spleen cells and thymocytes *in vitro* (Florentin *et al.*, 1979; Giroud *et al.*, 1983); similarly with medium from peritoneal exudate macrophages grown in culture (Calderon *et al.*, 1975; Calderon and Unanue, 1975). Lymphocyte proliferation has also been observed within the peritoneal cavity after subcutaneous injection of thioglycollate, although it was unclear whether cells within the peritoneal cavity were stimulated to divide, or whether they were stimulated and commenced DNA synthesis in some extraperitoneal site, and migrated into the peritoneum while in S phase (Yoffey and Yaffe, 1985).

A second possibility is that the activated lymphocytes *were* derived from the cellular exudate, in which case MPR-300 expression could well correlate with their ability to migrate into the peritoneal cavity. In spite of the low numbers of exudate lymphocytes observed here and by Beelen & Walker (1983), other investigators have demonstrated the entry of circulating lymphocytes into the inflamed peritoneal cavity , and shown that recently activated lymphocytes enter this site more readily than resting cells do. After labelling proliferating lymphocytes *in vivo* with [^{3}H]-thymidine, total lymphocytes collected from the thoracic duct of healthy rats (Beacham and Danielle, 1982; Koster and McGregor, 1970), or rats infected with the intracellular bacterium *L. monocytogenes* (Koster and McGregor, 1971; Koster *et al.*, 1971), were injected intravenously into recipient rats with induced peritoneal inflammation. Large proliferating lymphocytes and their smaller progeny entered the inflamed peritoneum more readily than did small, non-proliferating cells. Lymphocytes entering the inflamed peritoneal cavity of *Listeria*-infected mice were also sensitive to the antimitotic drug vinblastine (North and Spitalny, 1974). Given this, it seems likely that the activated lymphocytes examined in the present study originated in the circulation, in an activated state, rather than arising *in situ* through non-specific activation. In fact, the small number of lymphocytes observed in the peritoneal exudate could then be due to a lack of activated cells in the circulation; intravenous injection of activated cells or prior immunization

of the rats might have seen a larger lymphocyte influx into the peritoneal cavity.

If activation is required for lymphocyte entry into the inflamed peritoneal cavity, then circulating lymphocytes with the capacity to migrate may already express MPR-300 on the cell surface, as proposed by Parish *et al.* (1990) and supported by the effect of Con A on rat spenic T cells. Although no MPR-300 was detected on peripheral blood lymphocytes, we did not examine these cells in an animal undergoing an inflammatory reaction, and the numbers of suitably activated cells, with the capacity to migrate, could be too low in a healthy animal to detect. However, stimulation of a MBP-specific T cell line with antigen does not induce the cell surface expression of MPR-300 (D. Willenborg, unpublished results), suggesting that antigen-driven activation, unlike Con A-stimulation, may be insufficient to induce redistribution of MPR-300 to the plasma membrane. This introduces the possibility that some aspect of the migration process itself induced the expression of MPR-300 on the elicited lymphocytes. It is accepted that after leukocytes adhere to endothelium, an "activation" process is triggered by interaction with endothelial adhesion molecules (E-selectin)(Lo *et al.*, 1991) or surface-associated chemoattractants such as IL-8 (Tanaka *et al.*, 1993b) and platelet activating factor (Zimmerman *et al.*, 1992), which converts integrins to a functionally active form, facilitating strong adhesion.

Similarly, interaction with endothelial cells could potentially stimulate a redistribution of lymphocyte MPR-300 to the plasma membrane, which can occur very rapidly, in as little as 5-15 min (Braulke *et al.*, 1989). Interaction with endothelial cells or ECM components could then induce expression of MPR-300 on the lymphocyte surface, just prior to being needed for basement membrane degradation. This scenario has considerable appeal, as there is no need for circulating lymphocytes to constitutively express the enzymes required for basement membrane degradation on the cell surface. This prompted an investigation, described in Chapter 5, to determine whether interaction with endothelial cells affects expression of MPR-300 on the plasma membrane of T lymphocytes.

4.5 SUMMARY

Activated T cells appear to express a more invasive phenotype than do lymphocytes in the resting state, and activation has been correlated with increased adhesion to endothelial cells, and enhanced secretion of hydrolytic enzymes. Parish *et al.* (1990) have proposed that activation also induces an increase in the cell surface expression of lysosomal enzymes, which may participate in degradation of the subendothelial basement membrane by extravasating lymphocytes. This could be achieved through an activation-induced increase in cell surface expression of MPR-300, with or without a concommitant increase in the intracellular expression of MPR-46.

Cell surface expression of MPR-300 was examined on resting and activated T cells from a variety of sources. While MPR-300 is constitutively expressed on the plasma membrane of most cell types, there was a clear exclusion of this receptor from the plasma membrane of lymphocytes from secondary lymphoid tissues and the peritoneal cavity, and leukocytes from rat and human peripheral blood, with the exception of human neutrophils. Expression of MPR-300 was induced on the surface of rat T lymphocytes by several activating stimuli. Stimulation of splenic T cells with Con A induced the appearance of MPR-300 in a time-dependent manner. Expression was highest after 3-4 days stimulation, and was declining by day 6. Similar treatment did not affect human peripheral blood T cells, however. MPR-300 was also detected on the plasma membrane of activated T cells present in a thioglycollate-induced inflammatory peritoneal exudate.

This study demonstrates that resting T cells do not express MPR-300 at the cell surface, but can be induced to do so by several activating stimuli. Expression of MPR-300 on the plasma membrane of Con A-stimulated rat cells demonstrates a positive correlation between cell surface expression of MPR-300, and the ability of the cells to enter the CNS, as Con A-stimulated spleen cells from rats sensitized with MBP can transfer EAE 3-4 days after their initial exposure to Con A. Lymphocytes from peritoneal exudates have also been shown to be capable of migration into inflamed tissues elsewhere in the body. This correlation between T cell activation, the ability to enter extravascular tissues, and expression of MPR-300 on the plasma membrane, is consistent with the hypothesis that activated T cells express an invasive phenotype which includes the enhanced expression of lysosomal enzymes at the cell surface.

CHAPTER 5

EFFECT OF ADHESION TO ENDOTHELIAL CELLS ON MPR-300 CELL SURFACE EXPRESSION BY HUMAN AND RAT T LYMPHOCYTES

5.1 INTRODUCTION

Leukocyte migration from the circulation into peripheral tissues is a complex process, involving three major steps (Section 1.5). Cells initially adhere to the vascular endothelium, pass through the endothelial monolayer, and then degrade the underlying basement membrane before passing into the interstitium. The adhesion step, which is conceptually similar for all leukocytes, involves sequential binding by members of the selectin and integrin families. Weak selectin-mediated interactions loosely tether leukocytes to the vascular wall, and subsequent integrin-mediated binding converts the transient interaction to strong adhesion (Kincade, 1993).

Although once viewed as passive bystanders during extravasation (Cronstein and Weissman, 1993), endothelial cells are now recognised as active participants in this process. Circulating leukocytes are able to adhere transiently to endothelium, but cannot extravasate into the tissues without some induced phenotypic change (Pardi *et al.*, 1992; Pober and Cotran, 1991a). Recent work has highlighted the importance of endothelial-derived signals in initiating such changes. Triggering of integrin-mediated adhesion provides a good example. Although constitutively expressed on leukocytes, integrins require cellular "activation" before they develop optimum functional activity (Anderson and Springer, 1987; Buyon *et al.*, 1988; Kishimoto *et al.*, 1989b; Smith *et al.*, 1988; Vedder and HArlan, 1988; Wright and Meyer, 1986). This increase in binding avidity probably results from a conformational change (Kishimoto *et al.*, 1989a; Lo *et al.*, 1989; Wright and Meyer, 1986). Activation of integrins can be triggered through many membrane receptors, of which at least 12 have been identified (Pardi *et al.*, 1992; Tanaka and Shaw, 1992). Some of these are preferentially expressed on different leukocyte subsets, providing a means of selectively involving those cell types in the adhesion cascade (Hogg, 1993).

It has become apparent that, in the case of circulating leukocytes tethered to the vascular wall, integrins are triggered by ligands present on the apposing endothelial surface. Endothelial cells stimulated with thrombin or histamine express platelet activating factor (PAF) on the surface (Defilippi *et al.*, 1993; Zimmerman *et al.*, 1990), which binds to a G protein-linked receptor on neutrophils, and "functionally upregulates" integrin-mediated adhesion to endothelium *in vitro* (Lorant *et al.*, 1991). On cytokine-stimulated endothelial cells, E-selectin itself enhances the activity of CD11b/CD18 on neutrophils (Lo *et al.*, 1991). Several members of the chemokine family of cytokines also trigger

integrin-mediated adhesion *in vitro*. IL-8 is chemotactic for both neutrophils and T cells (Larsen *et al.*, 1989; Matsushima and Oppenheim, 1989), is produced by endothelial cells stimulated with TNF, IL-1β, or LPS (Streiter *et al.*, 1989), and binds to endothelium *in vivo* (Rot, 1992). *In vitro* studies have shown that IL-8 upregulates the expression and binding avidity of β_2 surface integrins on neutrophils (Detmers *et al.*, 1990; Huber *et al.*, 1991). Triggering of lymphocyte adhesion has been demonstrated with macrophage inflammatory protein-1β (MIP-1β), which induces adhesion of CD8 T cells to endothelial VCAM-1 via β1 integrins (Tanaka *et al.*, 1993a; Tanaka *et al.*, 1993b; Tanaka *et al.*, 1993c). MIP-1β has been detected on endothelium in lymphoid tissue and at sites of inflammation, (Tanaka *et al.*, 1993a), and is believed to bind to proteoglycan glycosaminoglycan sidechains on the endothelial cell surface (Tanaka *et al.*, 1993a; Tanaka *et al.*, 1993b). Tanaka & coworkers have proposed that endothelial presentation of pro-adhesive cytokines may be a general mechanism in the triggering of leukocytes.

Endothelial cells are clearly capable of interacting with adherent leukocytes in a manner which enhances their capacity to extravasate, as described above, by presenting an activating signal which increases the affinity of leukocyte integrins for their endothelial ligands. Expression of MPR-300 on the plasma membrane was proposed by Parish *et al.* (1990) to contribute to the ability of extravasating lymphocytes to degrade subendothelial basement membrane, by providing a means of attaching lysosomal enzymes to the cell surface. In the previous chapter, MPR-300 was shown to be inducible on the surface of rat T cells by several activating stimuli. In a manner analogous to the "functional activation" of leukocyte integrins, interaction with endothelium could also provide a stimulus for the upregulation of MPR-300 on the lymphocyte cell surface. The aim of this chapter was to investigate this possibility.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Preparation of leukocyte suspensions

5.2.1.1 Human leukocytes

Human peripheral blood leukocytes were prepared as described in Section 4.2.1. Activated T cells were prepared by stimulation with Con A or anti-CD3. Con A-stimulated cells, prepared as described in Section 4.2.5, were supplied by Dr. Hilary Warren (Woden Valley Hospital, ACT, Australia). In most experiments, they were used 6 days after initial exposure to Con A. Anti-CD3-stimulated T cells were activated on anti-CD3 coated plates (Taub *et al.*, 1993). Mononuclear cells isolated from human blood (Section 4.2.5) were resuspended in 10% FCS/RPMI medium at a concentration of 4 x 10^6 cells/ml, and incubated in plastic dishes coated with OKT3 mAb for 8 h at 37°C. Petri dishes (100 mm) were prepared by coating overnight at 4°C with 10 ml of sheep anti-mouse Ig antibody (Silenus, Hawthorne, Australia), diluted to 10 µg/ml in PBS. Plates were washed four times with PBS, once with 1% FCS in PBS, then incubated with OKT3 culture supernatant diluted 1:10 with 1% FCS/PBS for 1 h at 4°C.

5.2.1.2 Rat splenic T cells

Mononuclear cells were isolated from rat spleen as described in Section 4.2.3. B cells were removed by adhesion to immobilized antibody specific for rat Ig. Petri dishes (10 cm) were coated with 10 ml of a rabbit anti-rat Ig antibody (Silenus, Melbourne, Australia), diluted to 20 µg/ml in PBS, by incubating overnight at 4°C. Plates were washed four times with PBS and once with 5% FCS/PBS. Spleen cells suspended in 5 ml of 5% FCS/PBS (up to 30 x 10^6 cells per plate) were added, and the plate centrifuged for 10 min at 200xg, then incubated on a flat surface for 30 min at 37°C. Non-adherent cells were removed, and the plate washed vigorously three times. The pooled non-adherent cells were centrifuged and resuspended in 10% FCS/RPMI medium.

Con A-stimulated spleen cells were prepared as described in Section 4.2.4.

5.2.2 Preparation of endothelial cell monolayers

5.2.2.1 Human umbilical vein endothelial cells

Human umbilical vein endothelial cells were prepared by the method of Jaffe *et al.* (1973), with some minor modifications. Umbilical cords were obtained from the Woden Valley Hospital Maternity Unit, ACT. After removing damaged portions, one end of the cord was cannulated with a size 10 foley catheter (Boston & Pacific Co., Boston, MA), and blood removed from the vein by perfusing with 100 ml PBS. After clamping the other end with forceps, the vein was infused with type IV collagenase (50 U/ml; Worthington Biochemical Corporation, Melbourne, Australia), and the cord incubated in a shaking waterbath at 37°C for 15 min. Endothelial cells were then flushed from the vein by perfusion with 20 ml of Medium 199 (Flow Laboratories, Irvine, UK) supplemented with 20% FCS and 60 U/ml garamycin ((Schering Corp., Kenilworth, NJ). The cells were centrifuged at 250xg for 10 min, and the pellet resuspended in 5 ml of fresh medium containing 20% FCS, 60 U/ml garamycin, 4 mM L-glutamine, 13.5 U/ml heparin (David Bull Laboratories, Melbourne, Australia) and 100 µl/ml endothelial cell growth supplement (Sigma, St. Louis, MO). The cells were cultured in a gelatin-coated (0.1%) 25 cm^2 tissue culture flask at 37°C in a 5% CO_2 atmosphere. Medium was replaced twice per week. Once confluent, cells were passaged using 0.1% trypsin and 0.1% EDTA in PBS. Cells from passage 1 were used in most experiments (approximately 7-12 days in culture).

5.2.2.2 Rat brain endothelial cells

Microvascular endothelial cells were obtained from rat brain using a modification of the method of Tontsch and Bauer (1989). Twelve day old rats were killed by CO_2 asphyxiation, and the cortices dissected and rinsed with cold sucrose buffer (0.32 M sucrose in PBS). Tissue was chopped into 1 mm cubes and homogenized in 3 volumes of ice-cold sucrose buffer, using a homogenizer with a loosely fitting pestle (7 strokes). The homogenate was diluted fourfold with cold sucrose buffer and centrifuged at 1000xg for 10 min at 4°C. The supernatant was discarded, along with the white layer of myelin in the upper part of the pellet. The remaining pellet was resuspended in the same volume of cold sucrose buffer and recentrifuged, and this procedure repeated a third time. The pellet was finally resuspended in the same volume of sucrose buffer, and centrifuged at 100xg for 1 min at 4°C. The supernatant (I) was reserved, and the pellet resuspended in sucrose buffer and centrifuged at 100xg

for 1 min at 4°C. The pellet was reserved (III) and the supernatant (II) pooled with supernatant I. The pooled supernatants were centrifuged at 340xg for 1 min at 4°C, the supernatant discarded, and the pellet (IV) reserved. Pellets III and IV were each resuspended in sucrose buffer (half the original volume), combined and centrifuged at 1000xg for 10 min at 4°C. The supernatant was discarded and the pellet washed with cold PBS. Microvessels were dissociated with type I collagenase (Sigma, St. Louis, MO; #C-0130) by shaking the resuspended pellet in 7 ml of collagenase solution (200 U/ml) for 30 min at RT. Cells were centrifuged at 200xg for 10 min at RT, and resuspended in Medium 199 supplemented with 20% FCS, 60 U/ml garamycin, 4 mM L-glutamine, 13.5 U/ml heparin and 100 µl/ml endothelial cell growth supplement (Sigma, St. Louis, MO). Cells were cultured in fibronectin-coated (10 µg/ml) 25 cm^2 flasks for 24-48 h, then washed with PBS and cultured in fresh medium until confluent. They were identified as endothelial cells by immunofluorescent flow cytometry, using a mAb specific for Factor VIII-related antigen (Dako, Carpinteria, CA). Cells were passaged using 0.1% trypsin-0.1% EDTA, and subsequent passages were grown in gelatin-coated flasks. Cells from passage 2 were used in all experiments (approximately 30-36 days in culture).

5.2.3 Adhesion of leukocytes to endothelial cell monolayers

Endothelial cells were grown to confluence in 25 cm^2 tissue culture flasks. Leukocytes were suspended in 5 ml of endothelial culture medium (Section 5.2.2.1) at densities ranging from $6x10^5$ to $1x10^6$ cells/ml, depending on the number of cells available, and incubated with the endothelial cells for 2 to 20 h. Control cells were incubated in fresh endothelial culture medium for the same period. In some experiments, leukocytes were also incubated with the spent medium removed from the endothelial cell cultures.

Non-adherent cells were removed from the flasks, which were then washed twice with PBS and the washings pooled with the non-adherent cells. Adherent cells were released by incubating the washed flasks with 0.4% EDTA/PBS (5 ml), reflecting the dependence of T cell-endothelial adhesion on divalent cations (de Bono, 1976). Flasks were incubated for 5 to 10 min at 37°C, and bound cells were released by gentle pipetting with a 5 ml pipette, the process being monitered by light microscopy. A considerable number of HUVE cells were also released by EDTA treatment, however T cells were released from rat brain endothelial cell monolayers without causing significant detachment of endothelial cells. Adherent, non-adherent and control leukocytes were centrifuged at 200xg for 3 min, resuspended in 1 ml of 10%

FCS/RPMI medium and counted using a haemocytometer. In preparations of adherent human cells, endothelial cells were distinguished from the leukocytes on the basis of size and appearance, and were not included in the cell count.

5.2.4 Stimulation of T cells with chemokines

T cells were prepared from rat spleen as described above (Section 5.2.1.2) and resuspended in endothelial cell medium at a density of 1×10^6 cells/ml. The chemokines MCAF, RANTES, MIP-1α and MIP-1β (Genzyme Corporation, Cambridge, MA) were added to the cells at concentrations of 50 ng/ml; IL-8 was added at 100 ng/ml. Cells were incubated for 2 or 4 h at 37°C. Cells were then washed twice in 10% FCS/RPMI and resuspended at a density of 10^7 cells/ml.

5.2.5 Flow cytometry

Leukocyte subsets in peripheral blood were distinguished by flow cytometry, on the basis of size (forward scatter) and internal granularity (side scatter). Endothelial cells in preparations of adherent human leukocytes were distinguished from leukocytes on the basis of their higher forward and side scatter. Ten thousand cells from each sample were collected, and cells from appropriate subsets analysed by setting polygonal regions around the population of interest.

Cell surface MPR-300, CD4 and CD8 were detected by immunofluorescent flow cytometry, as described in Section 4.3.7.

In order to compare the results of separate experiments, expression of MPR-300 was quantified as an "MPR expression index" (MEI), this being the ratio between the median fluorescence intensity due to MPR-300 staining and that due to an appropriate isotype control antibody. Mean MEI values for different treatments were compared using Student's t test (Swinscow, 1978). p values less than 0.05 were considered statistically significant.

5.3 RESULTS

5.3.1 Effect of adhesion to endothelial cells on cell surface expression of MPR-300 by human peripheral blood leukocytes

As previously shown (Section 4.3.2), human peripheral blood lymphocytes and monocytes do not express MPR-300 on their cell surface, while neutrophils do. In this study, MPR-300 expression by leukocytes was assessed after a 4 h incubation with confluent HUVE cell monolayers. On average, 30% of peripheral blood leukocytes adhered to the HUVE cells in this time, and the percentages of adherent cells within each subpopulation are summarized in Table 5.1. Cell surface expression of MPR-300 on adherent, non-adherent and untreated populations was assessed by immunofluorescent flow cytometry (Fig. 5.1). Adhesion to endothelial cells did not affect MPR-300 expression on lymphocytes and monocytes, as neither adherent or non-adherent populations differed significantly from untreated control cells. Expression on adherent neutrophils was reduced compared to control levels, however this decrease was not significant at the 5% level.

5.3 2 Effect of adhesion to endothelial cells on cell surface MPR-300 expression by Con A-stimulated human T lymphocytes

As adhesion to endothelial cells did not induce MPR-300 expression on resting T cells, Con A-stimulated T cells were examined to see whether pre-activation made them more responsive to interaction with endothelium. Incubation of activated T cells with HUVE cell monolayers for varying periods resulted in adhesion of up to 50% (Table 5.1). Cell surface expression of MPR-300 on adherent, non-adherent and control T cells was assessed by immunofluorescent flow cytometry, and representative fluorescence histograms illustrating the effect of a 4 h incubation are shown in Fig. 5.2. Adherent cells clearly expressed MPR-300 on the cell surface, non-adherent cells showed a slight expression, while control cells showed none.

This effect was not reproduced in all experiments. However, blood from a number of donors was used in the preparation of Con A-stimulated T cells, and yet more donors of umbilical cords in the preparation of HUVE cells. Considerable variation was found between T cell donors: of four donors used in a total of nine experiments, T cell preparations from two showed increased expression of MPR-300 after adhesion to HUVE cell monolayers. Variation in HUVE cell donors may have contributed to variable results also, as one of the

Table 5.1

Leukocyte adhesion to cultured endothelial cells

Leukocyte	Incubation time (h)	Adherent cells (%)[a]	Replicates (n)
Human peripheral blood leukocytes[b]	4	30±4	—
- lymphocytes		26±4	5
- monocytes		67±6	5
- neutrophils		21±3	3
Con A-stimulated human T cells[b, c]	2	50±5	4
	4	36±6	9
	8	39±10	4
	20	41±2	3
Rat splenic T cells[d]	2	10±1	6
	4	8±2	5
	8	3.1	1
Con A-stimulated rat splenic T cells[d, e]	2	40±12	3
	4	39±6	3
Con A-stimulated rat splenic T cells[d, f]	2	58±7	3
	4	54±8	3

[a] Adherent cells were calculated as a percentage of the total number of cells recovered from each flask.
[b] Cells incubated with HUVE cell monolayers.
[c] Stimulated for 6-8 days.
[d] Cells incubated with rat brain endothelial cell monolayers.
[e] Stimulated for 3 days.
[f] Stimulated for 6 days.

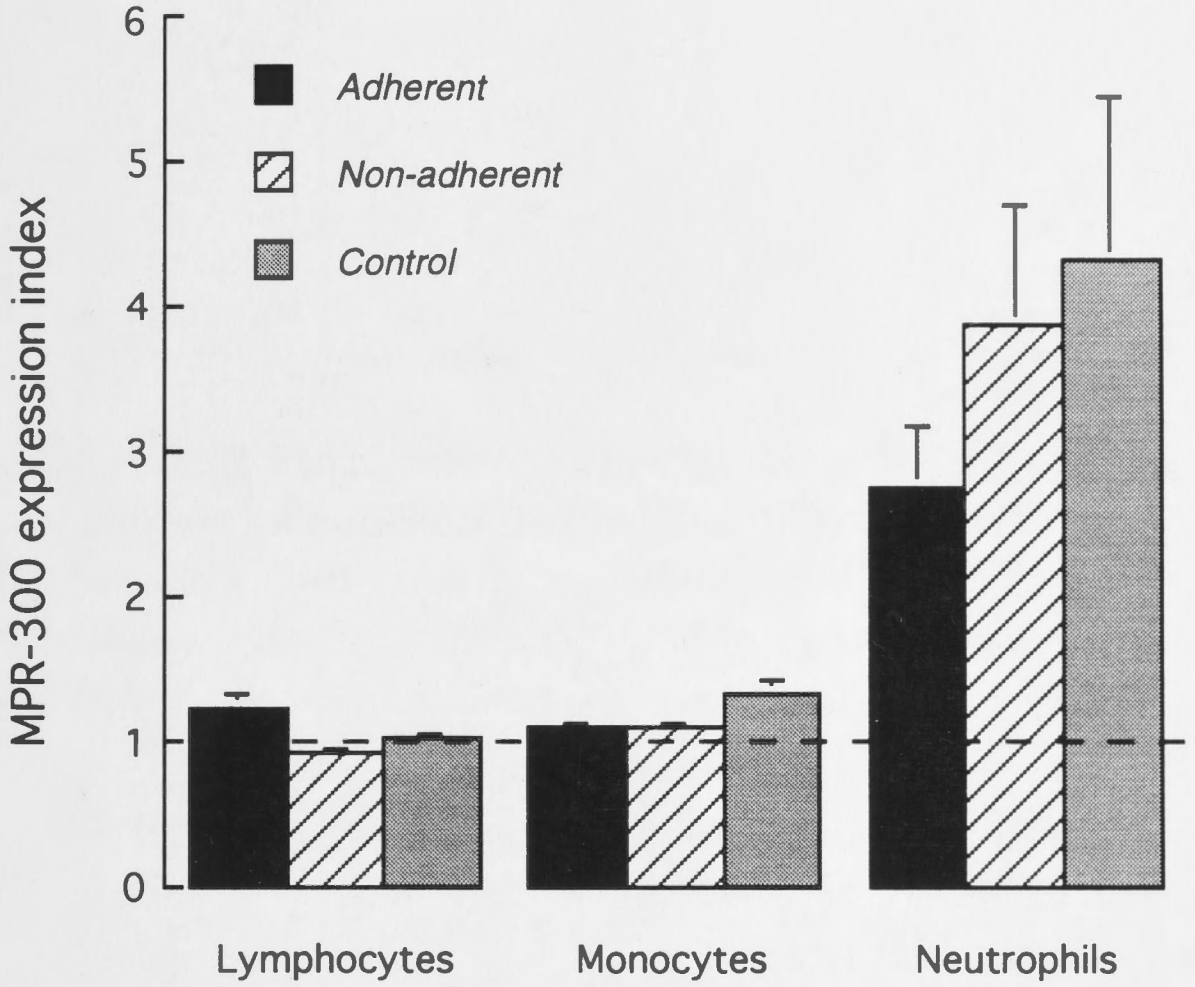


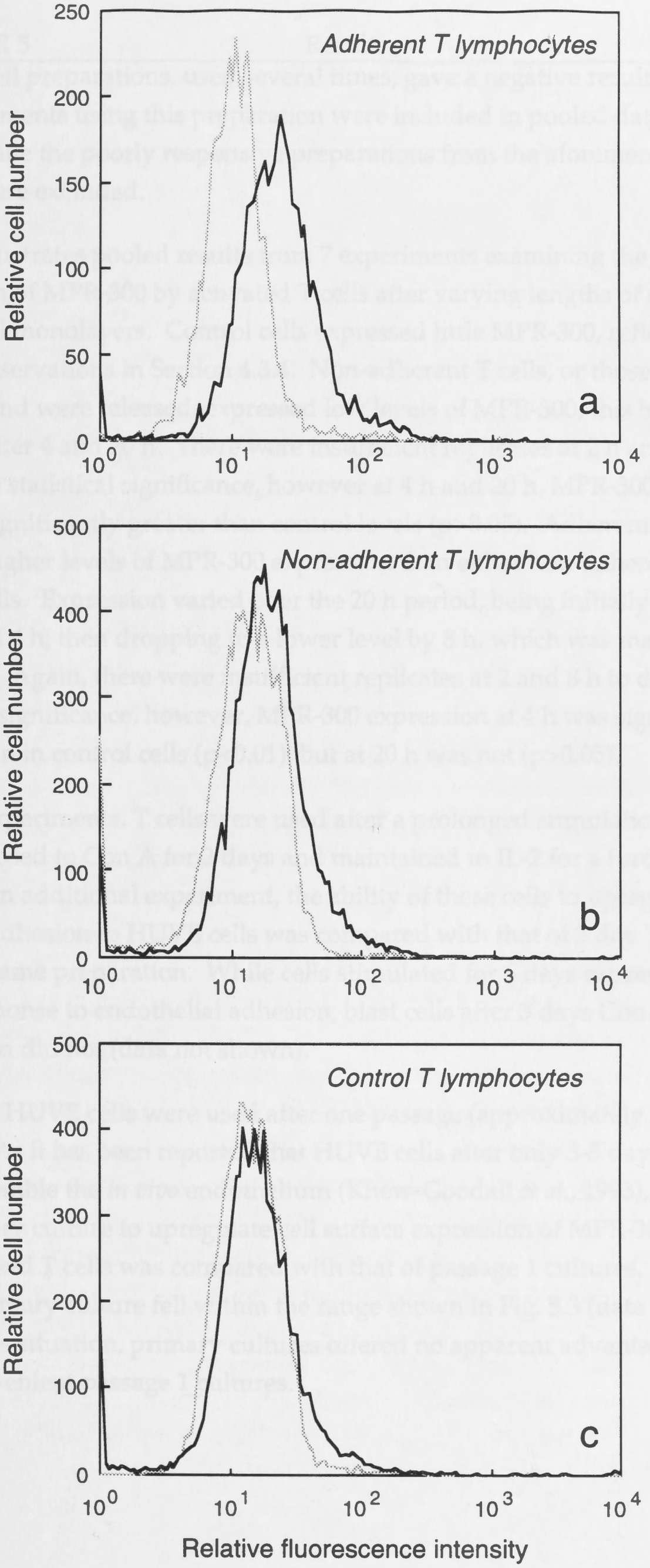
Fig. 5.1

Effect of adhesion to endothelial cells on cell surface expression of MPR-300 by human peripheral blood leukocytes. Leukocytes isolated from venous blood were incubated with HUVE cell monolayers for 4 h at 37°C. Adherent and non-adherent cells were obtained as described in Section 5.2.3, and compared with untreated leukocytes. Expression of MPR-300 was measured by immunofluorescent flow cytometry. Leukocyte subpopulations were distinguished on the basis of forward and side scatter (Section 4.3.1). Results are expressed as the ratio between staining due to rabbit antiserum (human MPR-300) and preimmune rabbit serum. A ratio of 1.0, represented by the dotted line, indicates no MPR-300 expression. Each value is the mean ± SEM (n=6).

Fig. 5.2

Cell surface expression of MPR-300 by Con A-stimulated T cells after exposure to endothelial cells. T cells isolated from human peripheral blood were stimulated as described in Section 4.2.5, and incubated with HUVE cells monolayers for 4 h at 37°C. Adherent (a) and non-adherent (b) cells were obtained as described in Section 5.2.3, and are compared with untreated T cells (c).

Expression of MPR-300 was measured by immunofluorescent flow cytometry. Binding of rabbit antiserum (——) was compared to staining due to preimmune rabbit serum (------).


Adherent T lymphocytes
a
Non-adherent T lymphocytes
b
Control T lymphocytes
c
Relative cell number
Relative fluorescence intensity
0
50
100
150
200
250
300
400
500
10^0
10^1
10^2
10^3
10^4

better T cell preparations, used several times, gave a negative result only once. All experiments using this preparation were included in pooled data described below, while the poorly responsive preparations from the aforementioned donors were excluded.

Fig. 5.3 illustrates pooled results from 7 experiments examining the cell surface expression of MPR-300 by activated T cells after varying lengths of exposure to HUVE cell monolayers. Control cells expressed little MPR-300, reflecting similar observations in Section 4.3.4. Non-adherent T cells, or those which adhered and were released, expressed low levels of MPR-300, this being greatest after 4 and 20 h. There were insufficient replicates at 2 h and 8 h to determine statistical significance, however at 4 h and 20 h, MPR-300 expression was not significantly greater than control levels ($p>0.05$). Adherent T cells showed higher levels of MPR-300 expression than either non-adherent or control cells. Expression varied over the 20 h period, being initially high, peaking at 4 h, then dropping to a lower level by 8 h, which was maintained until 20 h. Again, there were insufficient replicates at 2 and 8 h to determine statistical significance, however, MPR-300 expression at 4 h was significantly higher than in control cells ($p<0.01$), but at 20 h was not ($p>0.05$).

In these experiments, T cells were used after a prolonged stimulation period, being exposed to Con A for 3 days and maintained in IL-2 for a further 3-5 days. In an additional experiment, the ability of these cells to upregulate MPR-300 after adhesion to HUVE cells was compared with that of 3 day T cell blasts from the same preparation. While cells stimulated for 6 days expressed MPR-300 in response to endothelial adhesion, blast cells after 3 days Con A stimulation did not (data not shown).

Confluent HUVE cells were used after one passage (approximately 7-12 days in culture). As it has been reported that HUVE cells after only 3-5 days in culture better resemble the *in vivo* endothelium (Khew-Goodall *et al.*, 1993), the ability of a primary culture to upregulate cell surface expression of MPR-300 on Con A-stimulated T cells was compared with that of passage 1 cultures. The results for the primary culture fell within the range shown in Fig. 5.3 (data not shown), thus in this situation, primary cultures offered no apparent advantage over the more convenient passage 1 cultures.

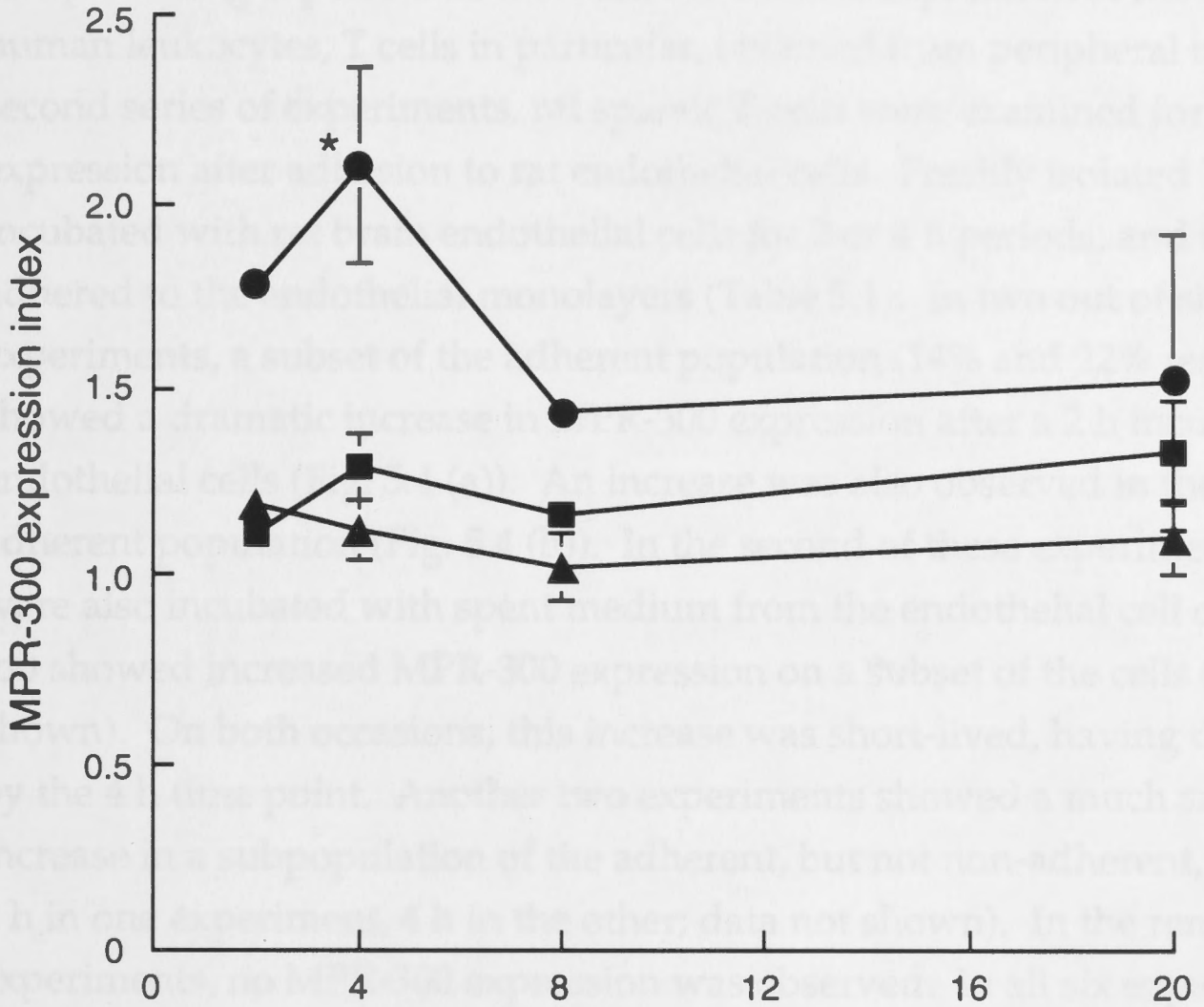


Fig. 5.3

Effect of adhesion to endothelial cells for varying lengths of time on cell surface expression of MPR-300 by Con A-stimulated peripheral blood T lymphocytes. Human T cells stimulated for 6 to 8 days (as described in Sections 4.2.5 and 5.2.1.1) were incubated for the indicated length of time with HUVE cell monolayers at 37°C. Adherent (●) and non-adherent (■) cells were obtained as described in Section 5.2.3, and were compared with control T lymphocytes (▲). Expression of MPR-300 was measured by immunofluorescent flow cytometry. Results are expressed as the ratio between staining due to rabbit antiserum (human MPR-300) and preimmune rabbit serum, such that ratio of 1.0 indicates no MPR-300 expression. Values represent mean ± SEM at 4 h (n=6) and at 20 h (n=3), and mean at 2 h and 8 h (n=2). Significant differences from control MEI values, calculated for 4 h and 20 h time points, are indicated by * ($p<0.01$).

5.3.3 Effect of adhesion to rat brain endothelial cells on cell surface MPR-300 expression by rat splenic T cells

The preceding experiments assessed cell surface expression of MPR-300 by human leukocytes, T cells in particular, obtained from peripheral blood. In a second series of experiments, rat splenic T cells were examined for MPR-300 expression after adhesion to rat endothelial cells. Freshly isolated T cells were incubated with rat brain endothelial cells for 2 or 4 h periods, and up to 10% adhered to the endothelial monolayers (Table 5.1). In two out of six experiments, a subset of the adherent population (14% and 22% respectively) showed a dramatic increase in MPR-300 expression after a 2 h incubation with endothelial cells (Fig. 5.4 (a)). An increase was also observed in the non-adherent population (Fig. 5.4 (b)). In the second of these experiments, T cells were also incubated with spent medium from the endothelial cell cultures; they too showed increased MPR-300 expression on a subset of the cells (data not shown). On both occasions, this increase was short-lived, having disappeared by the 4 h time point. Another two experiments showed a much smaller increase in a subpopulation of the adherent, but not non-adherent, T cells (after 2 h in one experiment, 4 h in the other; data not shown). In the remaining two experiments, no MPR-300 expression was observed. In all six experiments, the negative subset of T cells exhibited only a slight increase in MPR-300 expression, which was not significantly different to control levels ($p>0.05$). These experiments suggest that interaction with endothelial cells has the potential to induce transient expression of MPR-300 on a subset of unstimulated rat splenic T cells, but that the conditions required for this were not always reproduced.

In contrast to human T cells, Con A-stimulated rat T cells (3 days and 6 days), which already express MPR-300 on their surface (Section 4.3.4), failed to show additional expression following exposure to endothelial cells. A brief (8 h) stimulation with Con A, itself insufficient to induce MPR-300 expression, also did not enhance the ability of endothelial cells to upregulate MPR-300 expression (data not shown).

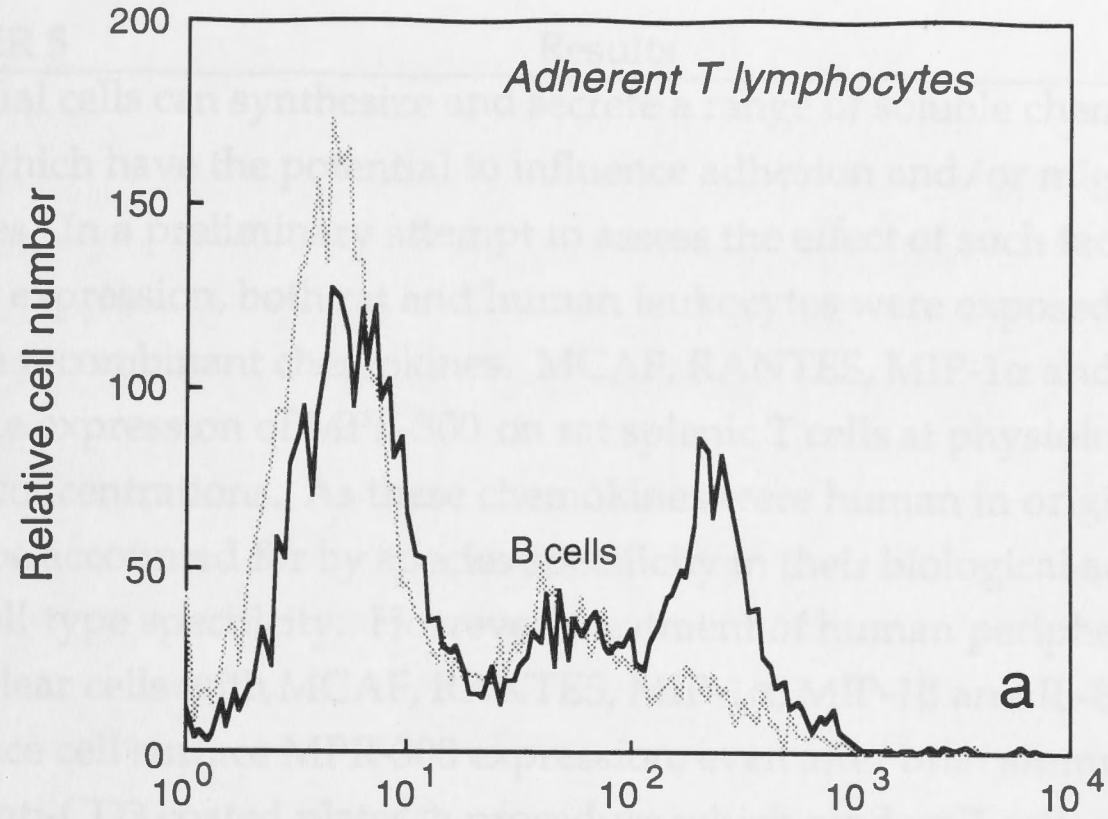
5.3.4 Effect of chemokines on cell surface expression of MPR-300 by human and rat leukocytes

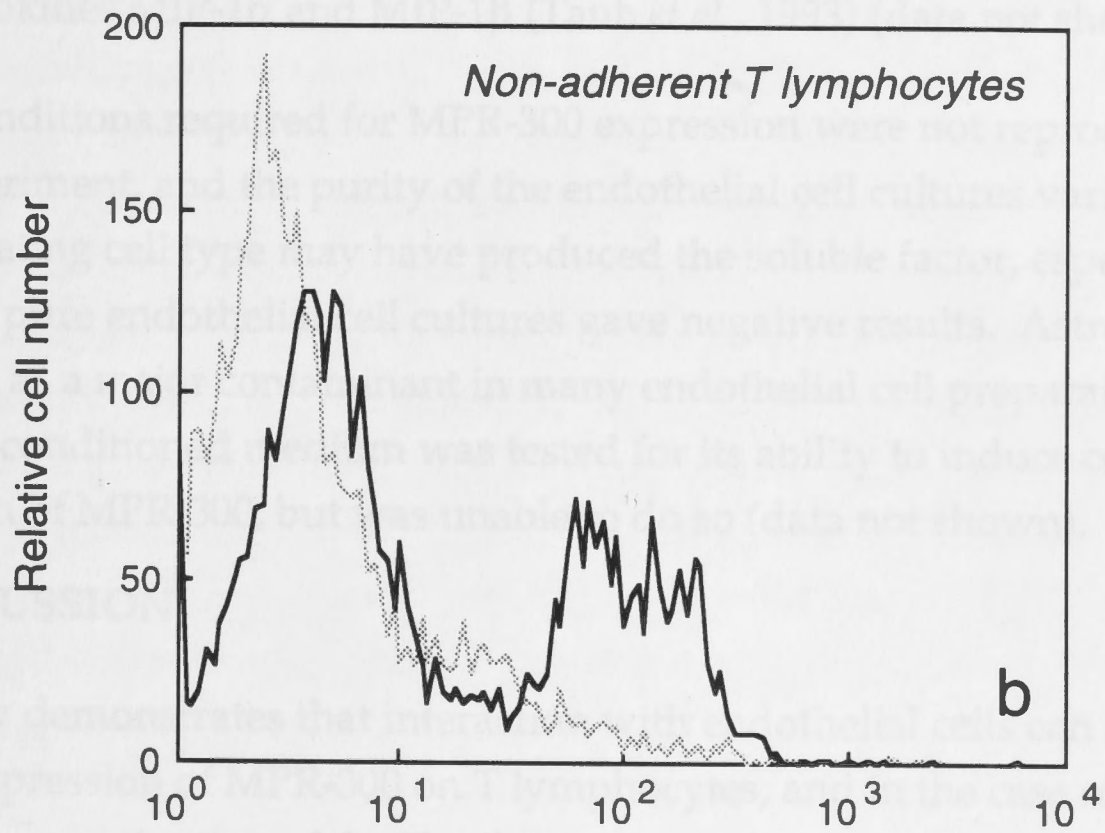
Upregulation of cell surface MPR-300 on rat splenic T cells by spent medium from endothelial cell cultures implied the involvement of a soluble factor, derived either from the endothelial cells or some other contaminating cell type.

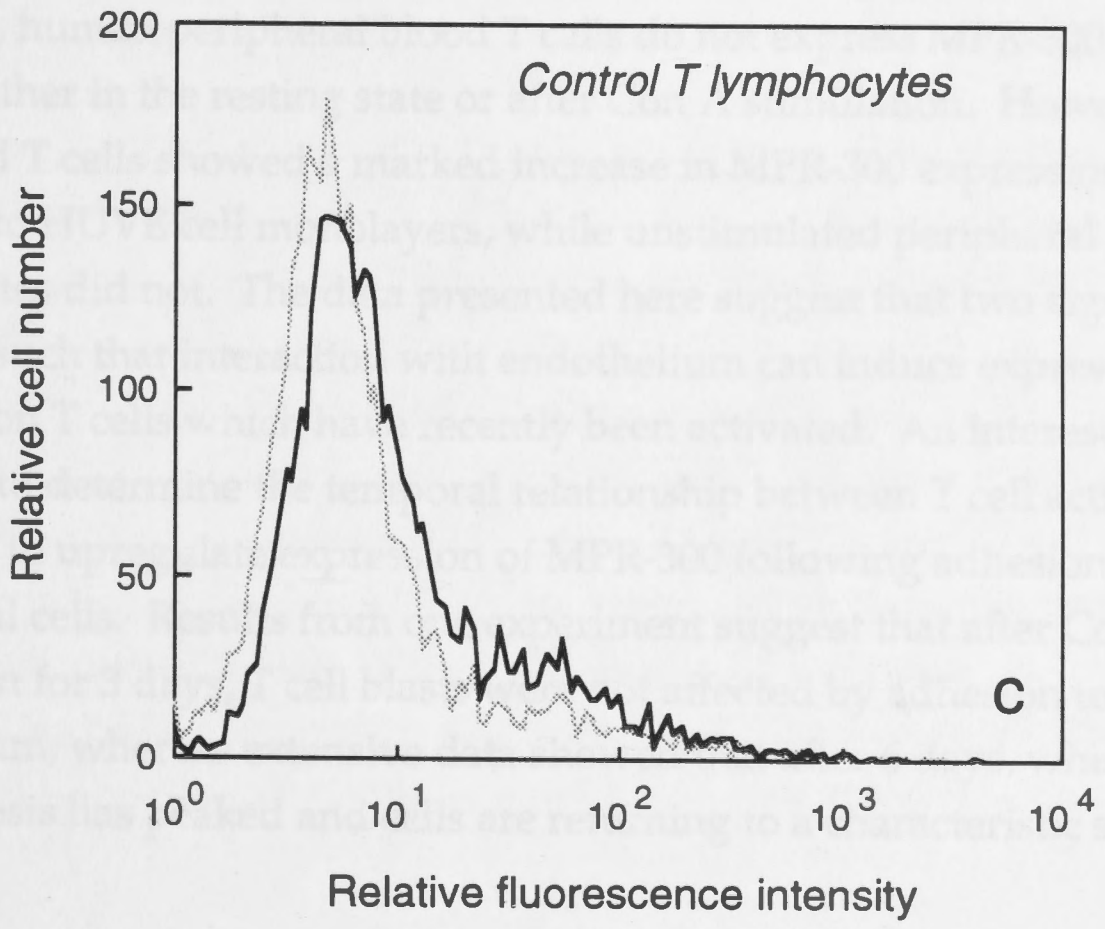
Fig. 5.4

Cell surface expression of MPR-300 by freshly isolated T cells following exposure to endothelial cells. T cells isolated from rat spleen were incubated with cultured rat brain endothelial cells for 2 h at 37°C. Adherent (a) and non-adherent (b) cells were obtained as described in Section 5.2.3, and are compared with untreated T cells (c).

Expression of MPR-300 was measured by immunofluorescent flow cytometry. Binding of mAb 1G7/9H4 (rat MPR-300) (——) was compared to staining due to an irrelevant mouse IgG_1 antibody (⋯⋯).


Adherent T lymphocytes
B cells
a
Relative cell number
200
150
100
50
0
10^0
10^1
10^2
10^3
10^4


Non-adherent T lymphocytes
b
Relative cell number
200
150
100
50
0
10^0
10^1
10^2
10^3
10^4


Control T lymphocytes
c
Relative cell number
200
150
100
50
0
10^0
10^1
10^2
10^3
10^4
Relative fluorescence intensity

Endothelial cells can synthesize and secrete a range of soluble chemotactic factors, which have the potential to influence adhesion and/or migration of leukocytes. In a preliminary attempt to assess the effect of such factors on MPR-300 expression, both rat and human leukocytes were exposed to a range of human recombinant chemokines. MCAF, RANTES, MIP-1α and MIP-1β did not induce expression of MPR-300 on rat splenic T cells at physiologically relevant concentrations. As these chemokines were human in origin, this could perhaps be accounted for by species specificity in their biological activity, as well as cell-type specificity. However, treatment of human peripheral blood mononuclear cells with MCAF, RANTES, MIP-1α, MIP-1β and IL-8 also failed to influence cell surface MPR-300 expression, even after brief stimulation of the cells on anti-CD3 coated plates, a procedure which renders T cells responsive to the chemokines MIP-1α and MIP-1β (Taub *et al.*, 1993) (data not shown).

As the conditions required for MPR-300 expression were not reproduced in each experiment, and the purity of the endothelial cell cultures varied, a contaminating cell type may have produced the soluble factor, especially as the two most pure endothelial cell cultures gave negative results. Astrocytes were identified as a major contaminant in many endothelial cell preparations; astrocyte-conditioned medium was tested for its ability to induce cell surface expression of MPR-300, but was unable to do so (data not shown).

5.4 DISCUSSION

This study demonstrates that interaction with endothelial cells can induce cell surface expression of MPR-300 on T lymphocytes, and in the case of human cells, that pre-activation of the T cells appears to be required. As shown in Chapter 4, human peripheral blood T cells do not express MPR-300 on the cell surface, either in the resting state or after Con A stimulation. However, Con A-stimulated T cells showed a marked increase in MPR-300 expression after adhering to HUVE cell monolayers, while unstimulated peripheral blood lymphocytes did not. The data presented here suggest that two signals are required, such that interaction with endothelium can induce expression of MPR-300 on T cells which have recently been activated. An interesting study would be to determine the temporal relationship between T cell activation and the ability to upregulate expression of MPR-300 following adhesion to endothelial cells. Results from one experiment suggest that after Con A stimulation for 3 days, T cell blasts were not affected by adhesion to endothelium, whereas extensive data showed that after 6 days, when blastogenesis has peaked and cells are returning to a characteristic small

lymphocyte morphology (Picker *et al.*, 1993b), cells *were* able to express MPR-300. T cells in peripheral blood, however, lacked this ability, although at least 50% demonstrate evidence of previous activation on the basis on CD45RO expression (Picker *et al.*, 1993b). This suggests a finite window of time during which T cells can respond to endothelial adhesion by expressing MPR-300 at the cell surface.

The ability of pre-activated T cells to express MPR-300 on the plasma membrane after adhesion to endothelial cells can be seen as evidence for, and an extension of the original hypothesis, which proposed that activation enhances the invasive potential of T cells by increasing the MPR-mediated expression of lysosomal enzymes at the cell surface. This study suggests that while activation is one requirement, an endothelial-derived signal is also needed to induce cell surface expression of MPR-300. This is an attractive possibility, as there is no need for circulating T cells to constitutively express the enzymes required for degrading the subendothelial basement membrane. Instead, cell surface expression of MPR-300, and consequently lysosomal enzymes, may be restricted to T cells in contact with the vascular endothelium. As discussed in Section 5.1, endothelial cells are capable of presenting "activating" signals to adherent leukocytes, which enhance their ability to extravasate, a clear example being the triggering of integrin-mediated binding. Upregulation of MPR-300 on adherent T cells could play an analogous role in enhancing their invasive capacity, such that circulating T cells adhere to endothelium, are induced to express MPR-300 on the plasma membrane, migrate beneath the endothelial cell layer where MPR-associated enzymes contribute to basement membrane degradation, and finally down-regulate expression of MPR-300 when it is no longer required.

The proposed endothelial-derived signal could induce expression of MPR-300 at the cell surface by stimulating either increased synthesis of the receptor, or a translocation from the intracellular pool to the plasma membrane. The latter has been demonstrated in response to extracellular signals in several cell types, including fibroblasts, adipocytes and hepatoma cells. Ligands for MPR-300 (M6P and IGF-II), growth factors (IGF-I and EGF), insulin and phorbol esters all induce a rapid and transient translocation of MPR-300, effecting a 1.5 to 4-fold increase in cell surface expression (Appell *et al.*, 1988; Braulke *et al.*, 1990; Braulke *et al.*, 1989; Corvera *et al.*, 1988; Hu *et al.*, 1990; Oka *et al.*, 1984; Oka *et al.*, 1985; Wardzala *et al.*, 1984). The mechanism by which this redistribution occurs is not understood, however it can be triggered by at least two signal

transduction pathways. M6P stimulates a pathway that is sensitive to cholera and pertussis toxins (Braulke *et al.*, 1989), while stimulation by IGF-II, IGF-I and EGF appears to involve protein kinase C (Braulke *et al.*, 1990). These studies illustrate that external stimuli can modulate the distribution of MPR-300, increasing its expression at the cell surface, and at least two signal transduction pathways can mediate this effect. G protein and protein kinase C pathways are activated by many receptor-ligand interactions, providing a potential mechanism for the redistribution of MPR-300 by a variety of stimulatory molecules. Thus, it seems quite plausible that an endothelial-associated molecule could influence the distribution of MPR-300 in adherent T cells.

Several differences were evident in the responses of human and rat T cells to both activation and interaction with endothelium. Like human peripheral blood lymphocytes, rat splenic T cells in the resting state did not express MPR-300 on the cell surface, however unlike human T cells, they *were* induced to do so by stimulation with Con A (Section 4.3), and adhesion of Con A-stimulated T cells to rat brain endothelium produced no further increase. Thus, while pre-activation and interaction with endothelium were required to induce MPR-300 expression on human T cells, Con A acting alone was able to provide rat T cells with the necessary stimulus. Con A is thought to activate cells by interacting with the TCR/CD3 complex, but also binds many other cell surface glycoproteins (Weiss, 1993). In binding to rat cells, Con A may mimic the endothelial-derived signal as well as supplying an activating signal. Adhesion of antigen-stimulated T cell clones to endothelium might help to clarify this. Antigen-stimulated cells do not express MPR-300 (D. Willenborg, unpublished observations), so the appearance of MPR-300 after adhesion would support the concepts that activation and endothelial-derived signals are required by rat T cells as well as human, and that the observed differences in rat and human T cell behaviour were due to different interactions with Con A.

On several occasions, when unstimulated splenic T cells were incubated with rat brain microvascular endothelial cells, a subset of the adherent cells expressed MPR-300 very strongly. This contrasts with the human T cell data, as interaction with HUVE cells failed to induce MPR-300 expression on unstimulated peripheral blood T cells. Picker *et al.* (1993b) have shown, however, that secondary lymphoid tissues, including the spleen, contain a small population of activated T cell blasts, which is absent from peripheral blood. The subpopulation of rat spleen cells which expressed MPR-300 after adhesion to endothelial cells may thus have been in an activated state.

Variation in the size of this activated population might also have contributed to the inconsistency of MPR-300 expression between experiments.

It is notable that rat cells expressed a much higher level of MPR-300 than did human cells, which again may reflect some difference in the T cell source, or in the source of endothelial cells. There are established differences between endothelial cells cultured from large vessels, and microvascular endothelium, as measured by cell surface markers (Gerlach *et al.*, 1985; Male, 1990 #522; McCarthy *et al.* 1991). In addition, brain microvessel endothelium differs from both large vessel endothelium and endothelium in other tissues; as well as the continuous tight junctions which contribute to the blood-brain-barrier, it expresses a variety of cell surface proteins not seen on other endothelia (Male *et al.*, 1990). The rat brain endothelial cells may then have provided a different or more effective stimulus to the T cells.

An obvious difference in the interaction between T cells and endothelium from rat and human was the nature of this association. While HUVE cells induced MPR-300 on human T cells by a method dependent on cell-cell contact, rat brain endothelium appeared to employ a soluble factor. This factor was secreted constitutively by cultured endothelial cells, as it was present in the spent medium of cells which had had no contact with T cells. In an *in vivo* situation, a secreted factor would presumably act at close range, as it would be rapidly diluted and washed away by the blood flow, a point that has been emphasized by Tanaka *et al.* (1993c). As these investigators demonstrated with MIP-1β, it may be presented to adherent cells while immobilized on the endothelial surface, however, in the static *in vitro* situation, sufficient of this factor was evidently produced to act on non-adherent cells as well.

Cultured endothelial cells can synthesize and secrete a range of soluble chemotactic factors, able to influence adhesion and/or migration of leukocytes, although this usually occurs in response to cytokine stimulation. These fall into two classes: the colony-stimulating factors GM-CSF and G-CSF (Broudy *et al.*, 1988; Broudy *et al.*, 1986; Munker *et al.*, 1986; Sieff *et al.*, 1987; Zsebo *et al.*, 1988), and several members of the chemokine family of cytokines. HUVE cells stimulated with TNF-α, IL-1β or LPS secrete IL-8 (Streiter *et al.*, 1989) and MCAF (also called MCP-1) (Sica *et al.*, 1990), while IFN-γ induces production of IP-10 (Duram and Oppenheim, 1993). Of these, only IL-8 and IP-10 are chemotactic for T lymphocytes (Duram and Oppenheim, 1993; Larsen *et al.*, 1989). Recombinant human IL-8, and several other commercially available chemokines were screened for their ability to induce MPR-300 expression on

both rat and human T cells, but were unable to reproduce the effect of the endothelial cell-derived factor. This is not unexpected, as unstimulated endothelial cell cultures were used in this study, while endothelial cells have only been observed to secrete chemokines after some form of activation.

The possibility remains that some contaminating cell type stimulated the endothelial cells to produce the soluble factor, or even produced it itself. The conditions required for induction of MPR-300 expression were not reproduced in all experiments, which supports the possibility of an interaction between endothelium and a second cell type, whose presence varied in different preparations. Astrocytes were identified as a contaminant in some of the rat brain endothelial cell cultures, however, astrocyte-conditioned medium did not reproduce the effect of the endothelial supernatant, indicating that astrocytes were not themselves the source of the soluble factor. They might, however, stimulate the endothelial cells to produce this factor by contact-dependent or -independent means. The latter could be tested in the adhesion assay by adding astrocyte-conditioned medium to a relatively astrocyte-free endothelial culture.

An important question which remains is *why* rat brain endothelium influenced T cells via a soluble mediator, when HUVE cells acted by a contact-dependent mechanism. No definitive answer is possible at this stage, other than to suggest that differences between the two types of endothelium may be responsible (Male *et al.*, 1990). Lymphocyte extravasation normally occurs in the microvasculature, not in large vessels, and so HUVE cells do not normally support lymphocyte traffic *in vivo*. The rat brain microvascular endothelial cultures may thus be more representative of endothelium in the postcapillary venules, and better able to provide appropriate stimuli to adherent lymphocytes. Therefore the soluble factor produced by the rat cells may well be the more physiologically relevant phenomenon of the two.

In conclusion, this study supports the proposal that interaction with endothelium provides one stimulus for the upregulation of MPR-300 on the T cell surface, as this receptor was expressed on human T lymphocytes after adhesion to cultured HUVE cells. This effect appeared to be limited to recently activated T cells, suggesting that two stimuli, activation and a second, endothelial-derived signal are required for expression of MPR-300 on the T cell surface. This study suggests that a modification to the Parish model is necessary, as activation alone appears insufficient to induce MPR-300 expression. Activation appears to prime the T cells in some way, such that after

they adhere to vascular endothelium, interaction with endothelial cells can induce MPR-300 expression on the T cell surface. In the case of the rat EAE model used by Parish *et al.* in developing their hypothesis, no modification is necessary as Con A-stimulated T cells were used to passively transfer EAE to naive recipients. According to the data presented in this study, these cells expressed cell surface MPR-300 prior to transfer, and so a second stimulatory signal would not have been required for their entry into the CNS.

5.5 SUMMARY

Endothelial cells are able to provide activating signals to adherent leukocytes, producing such effects as integrin-mediated tight binding, which enhance their capacity to extravasate and enter extravascular tissues. In this chapter, endothelial cells were examined for their ability to induce MPR-300 expression on the cell surface of adherent T cells. Expression of MPR-300 on the surface of human peripheral blood T cells, both resting and Con A-stimulated, was assessed after adhesion to HUVE cell monolayers. Neither cell type expressed MPR-300 initially, and adhesion to endothelial cells had no affect on resting peripheral blood T cells. Con A-stimulated cells, however, showed a marked, transient increase in MPR-300 expression at the cell surface. This effect was maximal after 4 h adhesion, and required cell contact. These data suggest that two signals are required to induce expression of MPR-300 on the T cell surface, such that interaction with endothelium provides a second signal to cells which have been pre-activated.

Some differences were evident in the response of rat splenic T cells to interaction with rat brain microvascular endothelial monolayers. A subpopulation of unstimulated T cells, possibly representing an *in vivo* activated population, expressed MPR-300 after co-incubation with endothelial cells, and this effect was mediated by a soluble factor. Treatment of rat and human T cells with the recombinant chemokines MCAF, RANTES, MIP-1α and MIP-1β had no effect on their expression of MPR-300, suggesting that these substances were not responsible. The soluble factor secreted by rat brain endothelial cells remains to be identified.

The response of rat T cells to endothelium further differed in that Con A stimulation itself induced expression of MPR-300, and interaction with endothelium produced no further increase. This may be reconciled with the behaviour of human peripheral blood T cells if the two cell types interact

differently with Con A, such that in binding to rat T cells, Con A mimics the endothelial-derived signal as well as supplying the pre-activating signal.

This study demonstrates that endothelial cells can upregulate the expression of MPR-300 on T cells, but suggests that this may be the second of two signals, the first in this case being supplied by prior stimulation with Con A. This suggests a modification to the hypothesis proposed by Parish *et al.* (1990), such that activation primes T cells in some way, enabling them to upregulate MPR-300 expression in response to an endothelial-derived signal. This is an attractive possibility as it correlates with the increased capacity of activated T cells to enter extravascular tissues, but restricts cell surface expression of MPR-300, and consequently lysosomal enzymes, to T cells already adherent to the vascular endothelium, thus limiting the expression of degradative activity to lymphocytes that have, or are about to, commence the process of extravasation.

CHAPTER 6

GENERAL DISCUSSION

6. GENERAL DISCUSSION

During the process of extravasation, leukocytes are thought to employ hydrolytic enzymes to degrade the sub-endothelial basement membrane, in a manner analogous to invasion by metastatic tumour cells. The ability of activated T cells to secrete serine proteases, as well as the endoglycosidase, heparanase, and of these to degrade the basement membrane in *in vitro* assays has been well documented. Following recent interest in the concept that the cell surface expression of degradative enzymes is important in the focussing and control of their activity in relation to locomotion of tumour cells through the ECM, Parish *et al.* (1990) proposed that extravasation of leukocytes may also depend on the cell surface expression, rather than secretion, of degradative enzymes. As extracellular lysosomal enzymes are known to bind to cell surface receptors (MPR-300), they proposed that basement membrane degradation by extravasating leukocytes may involve the cell surface expression of lysosomal hydrolases. In support of this hypothesis, they demonstrated that exogenous M6P, which displaces lysosomal enzymes from cell surface MPR-300, and CS, an inhibitor of oligosaccharide processing which prevents formation of the lysosomal enzyme M6P recognition marker, are both effective inhibitors of passively induced EAE and adjuvant arthritis in rats, diseases in which the initiation of inflammation is dependent on the passage of transferred T lymphocytes from the circulation into extravascular tissues. The aim of this thesis was to examine the hypothesis, proposed by Parish *et al.* (1990), that extracellular lysosomal enzymes bind to MPR-300 at the cell surface of extravasating leukocytes, and contribute to their ability to degrade the subendothelial basement membrane.

6.1 Specific inhibition of ligand binding to MPR-300

Chapters 2 and 3 describe an attempt to duplicate the anti-inflammatory behaviour of M6P, evident in its effect on T-cell mediated EAE and adjuvant arthritis in rats, using mAbs specific for MPR-300. A panel of 12 mAbs with specificity for the extracellular portion of MPR-300 were produced, and screened for their ability to inhibit the binding of M6P-bearing ligands to the receptor. None proved to have neutralizing activity, however. Although the display of an anti-inflammatory effect by an antibody able to neutralize the MPR-300:lysosomal interaction would have provided convincing support for this hypothesis, it was not possible to pursue this aspect of the project.

6.2 The effect of activation on cell surface expression of MPR-300 by T cells

Activated T lymphocytes are more active than resting cells in their ability to leave the circulation and enter non-lymphoid tissues, as T cell blasts more readily enter the CNS (Hickey *et al.*, 1991; Ludowyk *et al.*, 1992), and are required for the adoptive transfer of EAE (Richert *et al.*, 1979). This can in part be explained by their increased adhesiveness for vascular endothelium (Oppenheimer-Marks *et al.*, 1990; Savion *et al.*, 1984), and locomotor capacity (Parrott and Wilkinson, 1981; Ratner *et al.*, 1988; Wilkinson, 1986), however the ability of T cells to induce EAE has also been correlated with heparanase secretion (Naparstek *et al.*, 1984; Fridman *et al.*, 1987; Willenborg and Parish, 1988), suggesting that an ability to degrade the subendothelial basement membrane may also contribute to the invasive phenotype. Parish and coworkers proposed that the activated, more invasive phenotype may also correlate with increased expression of lysosomal enzymes at the cell surface, and thus with an increased expression of their cell surface binding sites.

In Chapter 4, the cell surface expression of lysosomal enzyme binding sites, in the form of MPR-300, was compared on resting and activated T cells. MPR-300 was not expressed on the cell surface of unstimulated lymphocytes, a notable absence in view of the seemingly ubiquitous expression of this receptor on the plasma membrane of all other cell types previously examined (excepting several transformed cell lines which do not express this receptor at all). In contrast, MPR-300 was expressed on the surface of rat splenic T cell blasts after 3-4 days Con A stimulation, and on an activated T cell subpopulation present in a thioglycollate-induced peritoneal exudate. Other investigators have demonstrated the invasive capacity of similar lymphocyte populations. Con A-stimulated spleen cells from MBP-immunized rats transfer EAE to naive recipients. As the cells are usually stimulated for 3 days prior to transfer, and enter the CNS within 24 h (Hickey *et al.*, 1991), they evidently possess a migratory phenotype 3-4 days after initiation of Con A stimulation, correlating with the appearance of MPR-300 on the cell surface. Lymphocytes derived from inflammatory peritoneal exudates are capable of entering inflamed tissues, migrating to DTH lesions and arthritic joints when injected into recipient animals (Issekutz *et al.*, 1986; Issekutz and Issekutz, 1991), and can also transfer contact sensitivity (Ptak and Asherson, 1969). This correlation between T cell activation and invasiveness, and expression of MPR-300 at the cell surface, is consistent with the proposal that activated T cells express an

invasive phenotype which includes the enhanced expression of lysosomal enzymes at the cell surface.

Several activating stimuli did not, however, increase the cell surface expression of MPR-300. Con A-stimulation did not affect its expression on T cells derived from human peripheral blood, and others have shown that it was not induced by antigen stimulation of a MBP-specific rat T cell line (D. Willenborg, unpublished observations). The latter express an invasive phenotype, as antigen-stimulated MBP-specific T cell lines transfer EAE even more effectively than do Con A blasts from immunized animals (Panitch, 1980; Richert *et al.*, 1981). Thus, while activation may enable lymphocytes to assume a more migratory phenotype, it does not necessarily directly induce MPR-300 expression at the cell surface, raising questions about the original proposal that MPR-300 expression is correlated with invasive, activated phenotype.

6.3 The effect of adhesion to endothelium on cell surface expression of MPR-300 by T cells

Expression of MPR-300 on the surface of peritoneal exudate lymphocytes suggested that the extravasation process itself might be involved in upregulating MPR-300 expression. The endothelium is known to have an activating effect on adherent leukocytes, enhancing their capacity to proceed with the extravasation process. Functional activation of integrins, and therefore the conversion of loose tethering to tight adhesion, is triggered by leukocyte interaction with endothelial adhesion molecules such as E selectin (Lo *et al.*, 1991), or with endothelial-associated chemoattractants (Tanaka *et al.*, 1993; Zimmerman *et al.*, 1992). The endothelium could thus potentially provide an MPR-300-inducing stimulatory signal to adherent T cells.

Chapter 5 describes an investigation of the effect of adhesion to endothelium on MPR-300 expression by T cells. The data presented in this study support the proposal that interaction with the endothelium results in MPR-300 expression on the T cell surface, with the qualifying requirement that the T cells be pre-activated. Con A stimulated T cells, derived from human peripheral blood, showed a marked though transient expression of MPR-300 after adhering to HUVE cell monolayers, while unstimulated T cells did not. This suggests that two signals are required to induce expression of MPR-300 on the surface of T cells: one an initial activation event, and the second derived from interaction with the vascular endothelium. Expression of MPR-300 on the surface of activated peritoneal exudate T cells, described in Chapter 4, is compatible with

this interpretation as these cells entered the peritoneal cavity from the circulation, and so have had the opportunity to respond to an endothelial-derived signal. The expression of MPR-300 on Con A-stimulated rat T cells, which suggested that activation alone could directly induce MPR-300 expression, can also be reconciled with this interpretation. As adhesion of Con A-stimulated cells to endothelium produced no further increase in MPR-300 expression, it may be that rat and human cells interact differently with Con A, such that it supplies rat cells with both the activating and otherwise endothelial-derived signals.

The data presented in this thesis are compatible with the proposal by Parish *et al.* (1990) that the invasive phenotype displayed by activated T cells translates to the expression of lysosomal enzymes at the cell surface, but suggest a modification to this hypothesis. Expression of MPR-300 does appear to be restricted to recently activated T cells, but activation alone, though necessary, may be insufficient to induce this change directly. A second signal, derived from the endothelium, is also required. This revised model is compatible with the physiology of the extravasation process, as circulating lymphocytes have no need to constitutively express enzymes required for degrading the subendothelial basement membrane. It seems plausible that circulating T cells express MPR-300 on the plasma membrane only after adhering to the vascular endothelium, just before it is required, thus establishing the machinery required to complete the extravasation process. Interaction with the basement membrane itself could likewise trigger a similar effect, by analogy with the inductive effects of fibronectin and laminin degradation products on proteinase expression by synovial fibroblasts and tumour cells, transduced through fibronectin and laminin receptors respectively (Turpeenniemi-Hujanen *et al.*, 1986; Werb *et al.*, 1989).

6.4 Possible contributions of the Con A- and endothelial-derived signals to upregulation of MPR-300 expression on T cells

What might be the relative contributions of the activating and endothelial-derived signals to the upregulation of MPR-300 on the T cell surface? The pre-activation step may prime the T cell in some way that enables it to respond directly and rapidly to a stimulus provided by the endothelium at a future time. The action of the endothelial-derived signal may resemble that of other external stimuli, including M6P, IGF-II and several growth factors, which have been shown to produce rapid increases in cell surface MPR-300 on fibroblasts by stimulating one of at least two signal transduction pathways, mediated by G

proteins (Braulke *et al.*, 1989) or protein kinase C (Braulke *et al.*, 1990b). These substances are thought to modulate the recycling of MPR-300 between the intracellular and cell surface pools, increasing the externalization rate while the internalization rate remains unchanged (Braulke *et al.*, 1990a; Braulke *et al.*, 1990b). As signal transduction pathways can be activated by many cell surface receptor-ligand interactions, an endothelial-associated molecule might also modulate the distribution of MPR-300 by activating one of these pathways.

Such a mechanism for the action of the endothelial-derived factor offers one explanation for the apparent requirement for T cell pre-activation. Resting T cells have a poorly developed cytoplasm, with few discernable organelles, and MPR-300 is scattered diffusely through small cytoplasmic vesicles, as well as in the cytosol itself (Olsen *et al.*, 1990). Lysosomal enzyme transport and intracellular movement of MPR-300 in resting T cells have not been examined, but very likely differ from that summarized in Fig. 1.15, given the poorly developed organelle system of these cells. If the endothelial-derived signal acts by modifying the intracellular movement of MPR-300 between the *trans* Golgi network, prelysosomal compartment and plasma membrane, then quiescent T cells may be physically incapable of responding. In contrast, activated lymphocytes possess highly developed cytoplasmic structures, including an extensive RER, conspicuous Golgi complexes, and many cytoplasmic vesicles. The distribution of MPR-300 resembles that of other cell types, being localized predominantly in the Golgi (Olsen *et al.*, 1990). Activation may therefore be necessary to establish MPR-300 recycling pathways between the Golgi/TGN and the prelysosomal compartment, which are then available for modulation by the endothelial-derived signal.

6.5 Is activation a requirement for T cell extravasation?

The requirement for pre-activation shown by human T cells suggests that, if lymphocyte extravasation does involve cell surface-bound lysosomal enzymes, movement into extravascular tissues is limited to recently activated T cells. T cells in most of the adhesion experiments were used 6-8 days after the onset of Con A stimulation. In a preliminary experiment, however, T cell blasts stimulated for only 3 days were not affected by adhesion to endothelium, suggesting a specific temporal relationship between activation and the ability to upregulate MPR-300 expression in response to an endothelial-derived signal. If this is correct, then our hypothesis suggests that activated T cells at the peak of blastogenesis are less capable of entering extravascular tissues than are cells

that have passed this peak and are returning to a small lymphocyte morphology.

Antigen recognition mainly occurs within secondary lymphoid tissues, and responsive lymphocytes undergo blastogenesis, which peaks 3-4 days after immunization. Some of these cells leave the lymph node, and have been observed in the thoracic duct 4-5 days after immunization. By day 6, most activated cells have either left the lymph node or reverted to small lymphocytes (Asherson *et al.* (1973) and references therein). This is consistent with the pattern of induction of MPR-300 expression on the lymphocyte surface: expression of MPR-300 and lysosomal enzymes is unnecessary on T cells at 3 days stimulation, as such cells *in vivo* are still located within the lymph node. It seems plausible that the ability to upregulate MPR-300 following adhesion to the vascular endothelium should be a characteristic of lymphocytes that have been released from the lymph node.

T cell blasts have, however, a clear ability to enter extravascular tissues. MBP-specific T cell lines stimulated with antigen for 3 days and injected intravenously, enter the CNS of the recipient within 24 h (Hickey *et al.*, 1991; Ludowyk *et al.*, 1992). T lymphoblasts generated *in vivo* in lymph nodes draining a site of antigen stimulation, and injected 3-4 days after immunization into syngeneic recipients, also accumulate in inflamed skin (Asherson *et al.*, 1973; Rose *et al.*, 1976). These experimental systems are not physiological, in that lymphoblasts are made available in the circulation in greater numbers than they would normally be, and at an earlier stage in their development. However, they clearly indicate that the mechanisms exist for entry of T lymphoblasts into extravascular sites, whether or not they are actually available in the circulation. As T cell blasts have the ability to migrate into extravascular tissues, then either (a) they do so independently of the proposed MPR-300/lysosomal enzyme mechanism; (b) the preliminary data, suggesting that 3 day T cell blasts do not express MPR-300 after adhesion to endothelium, is incorrect; or (c) as activated T cells probably acquire the ability to express MPR-300 at the cell surface at some point between 3 and 6 days stimulation, the time elapsed between the isolation of blast cells and their entry into extravascular tissues in the recipients was sufficient to allow the expression of MPR-300. To resolve this question, it will be necessary to explore more thoroughly the ability of activated T lymphocytes to respond to the endothelial-derived signal with increased expression of MPR-300, over the period of blastogenesis.

Entry of T cells into inflammatory sites, particularly those involving DTH reactions or chronic inflammation, involves small, recirculating lymphocytes as well as lymphoblasts (Parrott and Wilkinson, 1981). While this seems inconsistent with the proposed requirement for recent T cell activation, the majority of small lymphocytes present in these lesions have more recently been shown to express the CD45RO$^+$ antigen (Kingsley *et al.*, 1988; Pitzalis *et al.*, 1988; Pitzalis *et al.*, 1987; Volpes *et al.*, 1991), and their presence has been demonstrated to result from the preferential migration of this subset into the lesion (Pitzalis *et al.*, 1991; Sterry *et al.*, 1990). For several years after the discovery that T cells can be divided into two relatively distinct populations based on their expression of two isoforms of the CD45 antigen (designated CD45RO and CD45RA in the human), the CD45RA$^+$ and CD45RO$^+$ populations were widely thought to represent maturation steps, being naive and memory T cells, respectively. Activation of CD45RA$^+$ T cells results in a phenotypic change to CD45RO$^+$, along with increased expression of adhesion molecules, MHC class II and IL-2 receptors, and it was assumed that long-lived CD45RO$^+$ memory T cells developed from activated T cells (Mackay, 1993). As several groups have demonstrated the reversion of CD45RO$^+$ T cells to the CD45RA$^+$ phenotype (Bell and Sparshott, 1990; Rothstein *et al.*, 1991; Warren and Skipsey, 1991), this cell surface antigen has come to be seen more as a marker for cellular activation than for memory (Bell, 1992; Lightstone and Marvel, 1993; Mackay, 1992; Mackay, 1993). It is likely that the CD45RO isoform indicates activated cells and their progeny, which may then revert to CD45RA$^+$ resting cells at a subsequent stage. Thus, the CD45RO$^+$ small lymphocyte population appears to be an intermediate stage between activated (CD45RO$^+$) and resting (CD45RA$^+$) T cells, while the CD45RA$^+$ population includes both naive and previously activated cells (Mackay, 1993).

According to this interpretation, the small CD45RO$^+$ T cells which preferentially enter inflamed lesions are previously activated cells, falling in the intermediate stage between T cell blasts and resting cells. As MPR-300 expression is inducible on T cells 6-8 days after the onset of Con A-stimulation, and possibly longer, migration of these small lymphocytes into inflamed sites could be compatible with our hypothesis if they include the most recently activated members of the CD45RO$^+$ population. In support of this, small CD45RO$^+$ T cells entering cutaneous suction blisters created over DTH lesions expressed higher levels of the CD45RO antigen than did peripheral blood T cells (Pitzalis *et al.*, 1991). As expression of CD45RO has been observed to decrease with time after activation (Warren and Skipsey, 1991), the blister cells

may represent a more recently activated population than in the blood as a whole. Masuyama *et al.*, (1992) also showed that the 1% of peripheral blood T cells able to migrate through an endothelial monolayer *in vitro* were recently activated, based on their expression of high levels of CD26, position in the cell cycle (G_1 phase) and their active synthesis of RNA. As a result, they proposed that the small subpopulation of peripheral blood T cells with the ability to adhere to endothelium and penetrate vessel walls *in vivo* must be activated to some extent. Thus, the small $CD45RO^+$ T cells which enter non-lymphoid tissues may represent the most activated subset of the $CD45RO^+$ subpopulation available in the blood, and hence may have the ability to upregulate cell surface MPR-300 expression after adhesion to endothelium. It would be informative to determine whether T cells retain the ability to express MPR-300 at the cell surface for an extended period after activation. If the hypothesis that lysosomal enzymes attached to cell surface MPR-300 are involved in basement membrane degradation proves to be correct, then the ability of T cells to upregulate cell surface expression of MPR-300 in response to endothelial adhesion could be an indicator of their ability to extravasate.

6.6 Binding of lysosomal enzymes to cell surface MPR-300

According to the hypothesis proposed by Parish and coworkers, cell surface expression of MPR-300 is not, of itself, sufficient to mediate basement membrane degradation. The presence of lysosomal enzymes in the extracellular space, with intact M6P markers and capable of activity under extracellular conditions, is also essential. This enzyme pool must also be large enough to saturate the constantly re-internalizing pool of cell surface MPR-300. It is not as yet known whether such a pool of lysosomal enzymes is available to extravasating T cells.

Lysosomal enzymes could be secreted by the extravasating cells themselves. In the case of T cells, this could be induced by either of the two activating signals required for upregulation of MPR-300 expression. Biosynthesis of lysosomal enzymes by T cells increases after activation (Olsen *et al.*, 1990). Hence, saturation of MPRs in the Golgi/TGN (the sorting site) could lead to secretion of lysosomal enzymes via the secretory pathway. Enzyme secretion could also arise from a change in the relative proportions of MPR-300 and MPR-46 in the Golgi/TGN. While both MPRs carry newly synthesized enzymes to the acidified prelysosomal compartment, MPR-46 has also been implicated directly in their secretion (Section 1.13). Thus, an increase in the proportion of MPR-46 at the sorting site, induced by activation and/or the endothelial-derived signal,

could enhance the secretion of lysosomal enzymes. The intracellular expression of MPR-46 was not examined in this study, as the attempt to produce mAbs to this receptor was unsuccessful. However, it would be interesting to compare the intracellular expression of MPR-46 in activated T cells before and after adhesion to endothelium. It is also possible that the increase in cell surface MPR-300 observed after binding to endothelial cells, which may have arisen from a diversion of intracellular receptors to the plasma membrane, altered the balance between MPR-300 and MPR-46 in the Golgi/TGN sufficiently to promote secretion of lysosomal enzymes. However, other studies suggest this is unlikely, as M6P, IGF-II, growth factors and other substances which induce a low level redistribution of MPR-300 to the cell surface do not affect lysosomal enzyme targeting (Braulke *et al.*, 1990a).

An extracellular pool of lysosomal enzymes could also be supplied by other cells in the local environment. Endothelial cells, for example, produce several matrix-degrading enzyme activities, including uPA, and collagenases, gelatinases and a stromelysin (Laug *et al.*, 1985; Menashi *et al.*, 1993), and a sulfatase (Bartlett *et al.*, 1995b). They may also secrete lysosomal enzymes, which would then be available to bind to cell surface MPR-300 expressed on extravasating T cells. It is feasible that secretion of lysosomal enzymes by endothelium could be induced by a cell contact-dependent mechanism, much as MPR-300 upregulation on T cells is induced. It is also potentially significant that PMA-stimulated microvascular endothelial cells undergo a rapid, 60% increase in cell surface MPR-300, an effect which is reversed on withdrawal of the stimulus (Hu *et al.*, 1990). At sites of inflammation, activated endothelium could itself bind extracellular lysosomal enzymes to cell surface MPR-300 and contribute to basement membrane degradation.

6.7 Relevance of this model to extravasation by other leukocytes

It is not clear from this study whether this model for T lymphocyte extravasation can be applied to other leukocytes. In the rat, neither circulating neutrophils nor monocytes expressed MPR-300, nor did either cell type within the peritoneal exudate. Bartlett *et al.* (1994) have recently shown, however, that M6P inhibited the thioglycollate-induced entry of neutrophils into the murine peritoneal cavity. Extravasating neutrophils, like lymphocytes, are subject to activating stimuli after adhering to the vascular endothelium, which trigger the functional activation of integrins and permit tight adhesion (Zimmerman *et al.*, 1992), and the possibility that neutrophil activation induces cell surface MPR-300 expression was discussed in Section 4.4. It is feasible then that cell surface

expression of MPR-300 could be induced transiently on adherent neutrophils, as it was on T lymphocytes. It would be worthwhile to investigate this possibility by examining the expression of MPR-300 on the plasma membrane of neutrophils after adhesion to endothelium.

Human peripheral blood monocytes did not express MPR-300 at the cell surface, and there is no evidence to suggest that M6P influences monocyte entry into extravascular tissues. Cells of this lineage are, however, capable of expressing MPR-300 at the cell surface, as it is expressed on rabbit alveolar macrophages (Shepherd *et al.*, 1984) and the U937 monocyte-like cell line (Bleekemolen *et al.*, 1988). As adherent monocytes also receive activating stimuli from endothelium (Beekhuizen and van Furth, 1993), it may also be worthwhile to examine expression of MPR-300 on adherent monocytes.

6.8 Future work

Studies presented in this thesis support the hypothesis, proposed by Parish *et al.* (1990), that extravasating lymphocytes degrade the basement membrane by means of cell surface-expressed lysosomal enzymes, as MPR-300 (the lysosomal enzyme receptor) was found to be inducible on the cell surface of recently activated T cells by an endothelial-derived signal. An important unanswered question is whether the increased expression of MPR-300 on adherent T cells was matched by secretion of lysosomal enzymes. Lysosomal enzymes with intact M6P markers could be secreted by the T cells themselves, or by other cells in the vicinity, such as endothelial cells. As MPR-300 is induced on T cells after adhesion to endothelial cells, it is possible that lysosomal enzyme secretion could be similarly triggered on either cell type by a contact-dependent interaction.

Another area in need of further study is the proposed requirement for both an activating and an endothelial-derived signal for the induction of MPR-300 expression on the T cell surface. While this appeared to be necessary for human T cells, rat T cells expressed MPR-300 after stimulation with Con A alone. As interaction with endothelial cells induced no further expression of MPR-300 on Con A-stimulated rat cells, this lectin was proposed to supply both signals to rat, but not human, T cells. Comparison of Con A with more physiological means of T cell activation should help to clarify whether activation alone is sufficient to induce MPR-300 expression on the rat T cell surface, and if it is not, whether a second, endothelial-derived signal is effective in doing so. It has already been shown that *in vitro* antigen-stimulation does

not induce MPR-300 expression on a MBP-specific rat T cell line (D. Willenborg, unpublished observations), and T cell blasts activated *in vivo* could also be examined. Activated T cells could be generated in lymph nodes by immunizing rats with a contact-sensitizing antigen (Asherson and Allwood, 1972), and lymph node cell suspensions examined for co-expression of MPR-300 and activation markers. Antigen-stimulated T cells from both sources could then be used in the *in vitro* endothelial cell adhesion assay, or examined for expression of MPR-300 after extravasation into an extravascular site. Koster and coworkers have demonstrated that T cell blasts enter the inflamed peritoneal cavity (Koster and McGregor, 1971; Koster *et al.*, 1971), thus antigen-stimulated, fluorescently-labelled T cells could be injected into rats with induced peritoneal inflammation, and their appearance in the peritoneal cavity monitored. Expression of MPR-300 on the cell surface after, but not before, entry into the peritoneal cavity would provide additional evidence that events during the extravasation process induce the expression of MPR-300 on pre-activated T cells.

Another area not addressed in this study is the effect of cytokine-stimulation on the ability of endothelial cells to induce MPR-300 expression of the surface of adherent T cells. Leukocytes enter inflamed tissues more readily than they do normal, non-lymphoid tissues, this being reflected in the increased expression by cytokine-stimulated endothelial cells of tethering molecules, such as the selectins, and chemotactic factors capable of triggering integrin-mediated binding. Cytokine-stimulated endothelial cells may thus prove more effective than unstimulated endothelium in inducing MPR-300 expression on adherent T cells.

Finally, identifying the endothelial-derived signal should prove to be an interesting study. In recent years, the vascular endothelium has been recognized as playing an active role in the extravasation process, expressing selectins responsible for the initial capture and rolling of leukocytes along the endothelium, and triggering the functional activation of leukocyte integrins. The effect of endothelial cells on the expression of MPR-300 by adherent T cells introduces the possibility that the vascular endothelium is also involved in triggering the expression of hydrolytic enzymes by adherent leukocytes, enabling them to degrade the subendothelial basement membrane and so continue their passage through the vascular wall.

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